

THE DIFFERENTIATION OF OSTEOGENIC CELLS
FROM BONE MARROW.

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by

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THE DIFFERENTIATION OF OSTEOGENIC CELLS FROM BONE MARROW PRECURSORS.

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ABSTRACT.

According to a current hypothesis there is a putative stromal stem cell in bone marrow, capable of giving rise to adipogenic, osteogenic, fibroblastic and reticular cell lines. An aim of this thesis was to obtain information about the hierarchy and potential for differentiation of these marrow stromal cell lines in young adult rabbits. Particular objectives were to determine the conditions required for expression of the adipocytic phenotype in vitro, to obtain cloned populations of adipocytic cells and to compare their potential for differentiation with that of populations derived from marrow fibroblastic colonies using an in vivo diffusion chamber (DC) assay.

Adipocytic colonies differentiated and grew to a limited size in medium supplemented with rabbit plasma, but attempts to isolate and expand them in this medium failed. On changing the serum supplement to FCS, adipocytic cells acquired a less differentiated morphology. There was a large increase in colony growth and cells were produced in sufficient numbers for the DC assay. 31 fibroblastic and 21 adipocytic colonies were isolated and expanded. Of these, 11 fibroblastic and 8 adipocytic colonies provided enough cells for implantation in DC's. 4 of the 11 fibroblastic and 3 out of the 8 adipocytic colonies formed bone in the chambers. It was concluded that cells which have differentiated in an adipocytic direction are able to revert to a more proliferative stage and subsequently to differentiate along the osteogenic pathway. Adipocytic and fibroblastic cells cultured in vitro from marrow have, with osteogenic cells, a common precursor in adult marrow.

Several phenotype specific differentiation markers have been identified for extra-medullary, but not marrow adipocytes. PAL422(AP2), a P₂-like myelin protein is a specific marker expressed during differentiation by a number of extra-medullary adipocytic cell lines. A further objective was to determine if PAL422(AP2) is expressed in marrow adipocytic cultures, and to assess its value as a marker of adipocytic differentiation in the marrow stromal system. Using western and northern blot analysis, expression of PAL422(AP2) was demonstrated in rat marrow cell cultures then localized to pre-adipocytes and young adipocytes by immunohistochemistry. Thus, PAL422(AP2) is of value as a differentiation marker of marrow adipocytes.

CHAPTER 1. BACKGROUND AND INTRODUCTION.

1.1: INTRODUCTION.

The mammalian skeleton is a dynamic tissue, subject to constant remodelling in response to mechanical and physiological stresses throughout life. Its principal constituent tissue, bone, is a specialized form of connective tissue characterized by a mineral phase consisting of a highly substituted crystalline calcium hydroxyapatite. This is deposited in a soft organic matrix consisting mostly of collagen type I but including collagen types III and V, and a heterogeneous group collectively known as the non collagenous proteins of bone (Fig 1.1). Although only present in trace quantities some of the latter exhibit biological activity and may be of great significance in skeletal homeostasis.

Fig 1.1. Composition of bovine cortical bone expressed as % by weight (from Vaughan 1981).

	Fresh Bone	Dry Bone
Mineral	68.8	75.7
Organic Material	22.1	24.3
Water	9.1	----
Collagen	19.5	21.5
Non-collagenous Protein	2.5	2.8

From an embryological perspective most osteogenic tissue is of mesenchymal origin, however, some cranial and facial bones are neural crest derivatives (Le Douarin, 1982). Bone may form in

one of two ways; firstly, a mineralized matrix may be deposited directly onto a fibrous anlage to form a membrane bone, alternatively, a cartilaginous framework is laid down which is subsequently replaced with osseous tissue - endochondral ossification. Intra membranous ossification is largely confined to the flat bones such as those of the clavicles, mandible or cranial vault, whilst the majority, including those of the limbs and vertebrae, form by endochondral ossification.

Once skeletal form is established, three mechanisms bring about bone growth. Growth persists at cartilaginous growth plates in the long bones or those of the cranial base until late adolescence. Sutural growth may occur at sites where membrane bones forming the cranial vault meet, and finally, growth may occur by a process of remodelling reflecting a predetermined and co-ordinated process of resorption and deposition along opposing bone surfaces. This process is, for example, important in allowing the expansion of the marrow cavity during long bone growth . In pathophysiological situations such as fracture repair (Ham & Cormack, 1979) or ectopic ossification (Connor, 1983), a cartilaginous framework is established which is subsequently replaced by bone in a process mirroring endochondral ossification. It is unclear how osteoblast recruitment and bone formation during growth, repair or pathological processes relate to bone turnover during normal skeletal homeostasis. Despite different embryological origins, we assume that osteoblasts and

osteoclasts from different anatomic sites behave in the same way. The mechanism of calvarial turnover is thus thought to be similar to that along the endosteal surfaces of a long bone.

At a local level, skeletal homeostasis in the adult is a cycle of bone resorption, followed by and tightly coupled to osteosynthesis. This requires highly co-ordinated activity on the part of the two main functional groups of bone cells, the osteoblasts and the osteoclasts. Osteoblasts and osteoclasts are thought to have a limited life span (Beresford, 1989; Owen, 1963; McCulloch and Heersche, 1988), thus, there must be a continual process of bone cell recruitment from a precursor cell pool throughout life. Furthermore, recruitment of cells from this precursor cell pool must be a highly regulated and co-ordinated process representing a balance between the demands of osteoclastic resorption and osteoblastic deposition.

In disease states this balance may be upset leading to skeletal deficiency or bony deformity. In Paget's Disease of Bone for example, the primary lesion is thought to be in the osteoclast; the disease is said to be osteoclast driven, and osteoclastic over activity leading to bone lysis is followed by compensatory osteoblastic activity leading to bony deformity and osteosclerosis (Singer & Mills, 1983; Revell, 1986).

If the underlying processes governing skeletal homeostasis in

health and disease are to be understood, an understanding of the process of bone cell recruitment is essential, and this, in turn, requires an understanding of their respective progenitors.

1.2: STEM CELLS AND PROGENITOR CELLS.

The simplest definition of a stem cell is that it is any cell with a high capacity for self renewal extending throughout adult life (Hall and Watt, 1989). Additionally, stem cells are considered to have the potential to undergo differentiation to form one or more cell types capable of assuming different functional roles. Differentiation has been defined as a quantitative change in the cellular phenotype secondary to the onset of synthesis of new gene products (Potten and Loeffler, 1990). It may be recognised by a change in cell morphology, by changes in enzyme activity or changes in protein synthesis or composition. During its life a cell may undergo several differentiation events. As cells become increasingly differentiated their proliferative potential is progressively lost until, when they are incapable of further proliferation, they become terminally differentiated. Cells which are partially differentiated are restricted in their proliferative potential and in their potential for phenotypic expression. These cells are termed committed progenitors to distinguish them from stem cells.

The study of stem and progenitor cells is difficult for a

number of reasons. Firstly, there are very few stem cells. It has been estimated , for example, that there are less than 5 haemopoietic stem cells (CFU-S) per 10^5 murine marrow cells (Metcalfe, 1984). In the epidermis, stem cells are confined to the basal layers and only about 10% have sufficient proliferative potential to form recognizable foci of new epidermis (Hall & Watt, 1989). In the small intestine there is evidence of a single pluripotential stem cell capable of giving rise to the four differentiated intestinal cell types, paneth, columnar, goblet and entero-endocrine cells. The stem cell compartment again is small and has been estimated to consist of less than 16 cells lying towards the base of the intestinal crypts (Potten et al, 1987). In marrow, precursor cells capable of giving rise to the supporting stroma occur with a frequency of about 1 in 10^5 total marrow cells (Friedenstein, 1976), and analysis of calvarial-derived osteogenic populations in culture have suggested that osteogenic precursors occur with a frequency of about 1 in 400 cells (Bellows & Aubin, 1989).

The second difficulty is that stem cells have few distinguishing biochemical or morphological features useful as markers. Finally, stem cells have an inherent propensity for differentiation in vitro, and it has been suggested that a specific growth factor produced by differentiated cells is an important molecule in the regulation of stem cell proliferation (Heath et al, 1990; Rathjen et al, 1990). Even if a pluripotential precursor giving rise to osteogenic tissue

could be isolated, it is likely that there would be some spontaneous differentiation leading to a population heterogeneous with respect to phenotype and differentiation stage.

Early work on osteoblast precursors was based on meticulous histological and autoradiographic observation of skeletal tissue and skeletal-derived organ cultures. With the development of tissue culture, in vivo and in vitro techniques have been devised to allow the observation of clonal populations of cells - populations derived from a single cell. Observation of cloned populations of cells allow conclusions to be drawn indirectly about their common progenitor (Aubin et al, 1990). Whether the precursor cell is unipotential or multipotential for example, or whether it has a large or small proliferative potential can be inferred to some extent by the size of the colony and the cell types present within it.

1.3: STROMAL STEM CELLS IN MARROW - THE STROMAL HYPOTHESIS.

The hypothesis suggesting a stromal stem cell system in marrow was proposed by Owen (1978; 1985). It stated that, there is, present in bone marrow, a putative stromal stem cell capable of giving rise to a number of differentiated phenotypes including the osteoblast, the chondroblast or chondrocyte, the adipocyte and a number of poorly characterised cell types including the fibroblast, myofibroblast or reticular cell. The

system may be represented by the lineage diagram shown in Fig 1.2. Moving down the diagram cells become progressively more differentiated. With increasing differentiation and maturation proliferative capacity declines. Although the lineage diagram suggests discrete stem cell, committed progenitor cell and differentiated cell stages, it is likely that the stem cell and the terminally differentiated cell represent the extremes of a range, with each progenitor lying at a different point somewhere in between. At one end of the range multipotential precursors with considerable proliferative potential are grouped together as stem cells, whilst at the other end, precursors with restricted growth and differentiation potential are described as committed progenitors or differentiated cells. It follows from this discussion that no two precursors are likely to be the same, and that each will differ in its proliferative and differentiation potential.

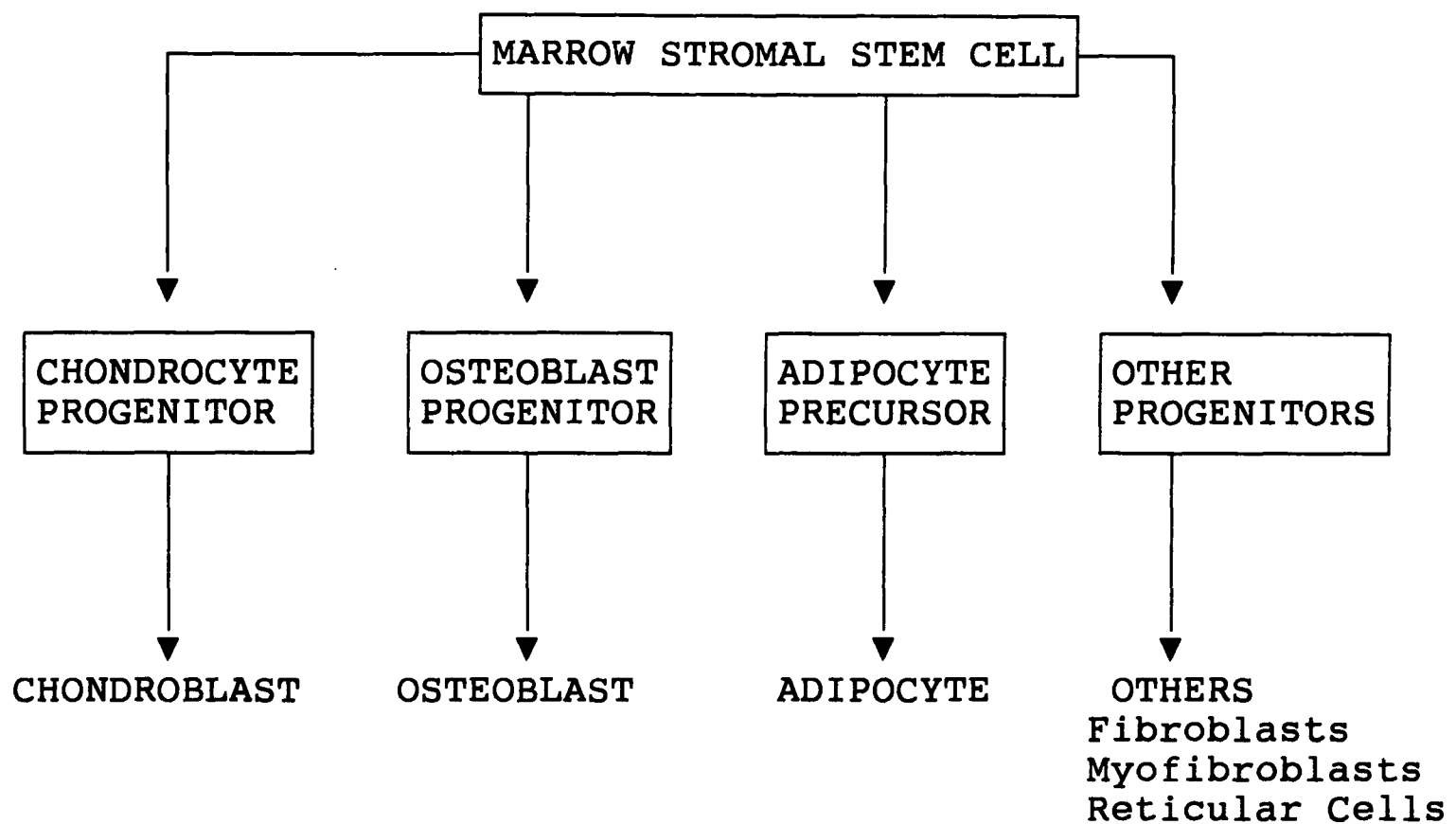


Fig 1.2. Diagram to illustate lineage relationships within the marrow stromal system, after Owen 1978; 1985.

In subsequent sections features of the differentiated stromal phenotypes will be discussed before progressing to a review of evidence in support of a common origin for osteoblasts, chondroblasts, adipocytes and the other stromal phenotypes in marrow, periosteal or endosteal derived stromal tissue.

1.4: BONE CELLS.

Differentiation in cell populations associated with the synthesis and maintenance of bone may be recognized at three levels. Cells may be identified at a microscopic level, by their anatomic position and the acquisition of characteristic osteoblast-like features, by metabolic changes such as changes in enzyme activity or the development of characteristic

responses to calcitropic hormones, and finally by changes in protein synthesis leading to the secretion of osteoblast derived extra-cellular matrix proteins. Four cell types are recognised, the osteoclast, the osteoblast, the osteocyte and the bone lining cell.

1.4a: THE OSTEOCLAST.

Osteoclasts are large multinucleated cells which resorb mineralized bone and cartilage. They are found on bone surfaces at sites of active resorption where osteoclastic activity leads to pits termed 'Howships lacunae', and a characteristic scalloped shaped appearance on histological sectioning. Two specialized features are commonly described. Adjacent to the site of resorption the plasma membrane is thrown into a series of folds giving a ruffled appearance, termed the 'ruffled border'. Peripherally, towards the margins of the cell the ruffled border gives rise to a 'clear zone', which forms a tight abutment against the surface of the bone, forming a sealed cuff around the ruffled border.

Recent attention has been drawn to the nature of the clear zone. A tight contact between the bone surface and the osteoclast is essential for resorption. Osteoclasts express two integrin receptors, members of a family of glycoprotein receptors capable of recognising a range of cell and extra cellular matrix associated ligands (Horton and Davies, 1989; Horton, 1990). These may be significant for cell matrix

interaction, and have been suggested as a possible target for therapeutic intervention in conditions characterized by excessive osteoblastic activity.

The origin of the osteoclast is uncertain. It is generally accepted to be a cell of the haemopoietic lineage. Although it has features in common with the macrophage, its development from the granulocyte/monocyte precursor cell remains controversial (Nijweide et al, 1990).

1.4b: THE OSTEOLAST.

Osteoblasts are found lining the bone at sites of osteosynthesis where they form a layer of cuboidal cells 15 μ m - 30 μ m wide, deep and high and continuous with the bone lining cells. Osteoblasts are asymmetric, synthesizing and secreting extra cellular matrix along one side (Weinstock and Leblond, 1974; Weinstock, 1979). Commonly, the nucleus is situated some distance from the matrix secreting surface. At an ultrastructural level, cells communicate with each other and with cytoplasmic processes of the osteocytes lying within the bone. At points of contact between osteoblasts and osteocytes gap junctions are found (Jee, 1988; Vaughan, 1981). This inter-cellular communication may be important in integrating osteosynthetic activity to allow remodelling of skeletal structures many times larger than an individual cell.

Tritiated thymidine studies have demonstrated that their

immediate precursors lie in the fibroblastic layer close to the active osteoblasts (Owen,1963) and that some may subsequently become incorporated into the matrix of bone to form osteocytes.

1.4c: THE OSTEOCYTE.

Osteocytes are the principal cells found within fully formed bones and lie in lacunae surrounded by matrix. Young osteocytes resemble mature osteoblasts and they originate from osteoblasts incorporated into the matrix during bone formation as the osteoid front advances. As they grow older cellular organelles disappear, cells come to occupy less space within the lacunae and become surrounded by a ring of non mineralized amorphous matrix. They are connected to each other by a network of canaliculi and are never more than 0.2mm from a matrix capillary (Ham and Cormack, 1979). Osteocytes are unable to divide and their function is unknown. They are, however, essential for the maintenance of vital bone and their loss is accompanied by tissue death.

1.4d: THE BONE LINING CELL.

These cells differ from active osteoblasts. They may be quiescent osteoblasts or alternatively might represent a separate bone cell population. They appear elongated and flattened like fibroblasts on histological sectioning, and probably form a contiguous layer lining the bone surfaces

(Vaughan, 1981). There are few organelles but long, thin cytoplasmic processes extend from the cell into the adjacent bone matrix. Their function is unknown. They may have a possible role in transducing mechanical or humoral stimuli into bony responses. It has been suggested that conformational changes in the lining cell layer in response to calcitropic hormones like parathyroid hormone (PTH) (Jones & Boyde, 1976) may expose the bone matrix to osteoclast action. Bone lining cells have been described as resting cells, yet, they take up ^3H Thymidine indicating DNA synthesis (Bowman and Miller, 1986; Kimmel and Jee, 1977) and may well be capable of further division and differentiation. Furthermore, it is unclear if they have the potential to become active osteoblasts.

1.5: FEATURES OF THE OSTEOLAST PHENOTYPE.

Studies on the differentiation of osteoblast-like cells in vitro, and extrapolation of the results to osteoblasts in vivo require stage specific markers of differentiation. To-date, studies have been restricted by the lack of suitable markers, however, a profile of the osteoblast phenotype is emerging and is discussed in subsequent paragraphs. For descriptive purposes markers of the osteoblast phenotype can be grouped into three categories including,

- i) enzyme markers,
- ii) cellular markers including monoclonal antibodies raised against specific cell surface antigens,
- iii) osteoblast derived matrix proteins.

1.5a: ENZYME MARKERS.

There are no known histochemical markers specific to the osteoblast, however, alkaline phosphatase (AP) is a plasma membrane bound enzyme (Doty and Scofield, 1976) of unknown function synthesized at varying levels by many cell types. Particularly high levels of the bone/liver/kidney isoform are found in osteoblasts, pre-osteoblasts and cartilage cells (Robison, 1923), and in association with matrix vesicles, thus in specific situations in vitro or in vivo such as adjacent to a bone surface or in cultures of osteoblast-like cells, alkaline phosphatase activity is an indicator of osteoblastic differentiation. AP activity varies widely and is maximal in young osteoblasts just prior to ossification (Pritchard, 1967). Once calcification begins there is a diminution of enzyme activity with very little in mature osteoblasts or osteocytes. Thus, AP activity is a characteristic of pre-osteoblasts or young osteoblasts.

Subsequently, alkaline phosphatase activity has been demonstrated in osteoblasts and their precursors at an immunocytochemical level (Bruder and Caplan, 1990a) and using cDNA probes to alkaline phosphatase message (Weinreb et al, 1990).

1.5b: CELLULAR MARKERS OF THE OSTEOLASTIC PHENOTYPE.

Specific humoral agents have osteosynthetic or osteolytic

effects in vivo. Parathyroid hormone (PTH) variously acts to promote osteolysis or osteosynthesis depending on the experimental conditions (Parsons, 1976). Osteoclasts are unable to respond to PTH in vitro, and the calcitropic response to the hormone is thought to be an indirect effect, mediated by soluble agents acting at a local level following stimulation of the osteoblasts by PTH (McSheehy and Chambers, 1986). Using radiolabelled PTH, receptors to the hormone have been found in many tissues including liver, kidney and bone. In connective tissues lining the bone surfaces they are confined to pre-osteoblasts or osteoblasts, and were not identified in osteocytes or osteoclasts (Rouleau et al, 1980; Silve et al, 1982). Conversely, osteoclasts have membrane receptors for, and respond to physiological doses of calcitonin (Warshawsky et al, 1980; Nicholson et al, 1986).

At an ultrastructural level, PTH has been shown to induce conformational changes in isolated osteoblast-like populations cultured on collagen gels (Jones and Boyde, 1976) and it has been suggested that these changes expose the underlying bone matrix to the resorptive action of the osteoclasts. Calcitonin causes the loss of ruffled borders in osteoclasts in calvarial organ culture (Holtrop et al, 1974), and inhibits their mobility in isolated osteoclast preparations, observations consistent with the inhibition of osteoclastic bone resorption.

Since the demonstration by Chase and Aurbach (1970) that PTH

increases cyclic AMP (cAMP) production it has generally been accepted that the bone resorbing effect of PTH is mediated by osteoblast cell surface receptors via an adenylate cyclase pathway and an increase in cAMP formation. Furthermore, osteoblast-like populations enriched for PTH responsiveness form bone when implanted in vivo in diffusion chambers (Simmons et al, 1982). The increase in cAMP formation in response to PTH has been used extensively to characterize osteoblast-like populations, osteosarcoma derived cell lines and adherent marrow stromal populations in vitro (Majeska et al, 1980; Martin et al, 1976; Partridge et al, 1981; Barling et al, 1989). Whilst the cAMP response to PTH is a useful marker of the osteoblast phenotype, biochemical assays of this sort require large numbers of cells and cannot be applied to cell populations in situ or in vivo.

A range of cytokines and growth factors found in the bone matrix or produced by bone or marrow cells are considered to be important local regulators of bone cell function. In vitro, they may be mitogenic for bone cells or regulate protein synthesis (reviewed by Canalis et al, 1989; Hauschka et al, 1988). Examples are given in Fig 1.3. Most are not unique to bone and are not of value when distinguishing osteoblasts from non osteoblast-like cells.

Epidermal growth factor is not found in the bone matrix and is not believed to be produced by bone cells, however, interesting results on its localization require discussion.

Autoradiographic studies on histological sections of rat femur and mandibular bone following perfusion with 125 Iodo-EGF localized EGF receptors to cells resembling pericytes (Martineau-Doize et al, 1988) which have been suggested as putative stromal precursors (Ham and Cormak, 1979). EGF receptor expression, however, was not apparent in cells close to the bone surface.

In vitro studies on intermediate released collagenase digest populations resembling earlier, less differentiated mesenchymal cells (Section 1.9a(i)) demonstrated high numbers of EGF receptors per cell, and an enhanced mitogenic response to EGF when compared to late released osteoblast-like populations (Van de Pol et al, 1984). When adherent stromal colonies which are considered to be osteogenic (Friedenstein et al, 1987) are grown in vitro in the presence of EGF the average colony size was greater whilst alkaline phosphatase expression decreased, i.e. proliferation increased whilst alkaline phosphatase expression decreased (Owen et al, 1987). It cannot yet be concluded that putative osteogenic progenitors in vivo or in vitro express EGF receptors, but it is possible to hypothesise that, in osteoblast-like populations, EGF receptor expression may be of value in distinguishing earlier progenitors from later progenitors or differentiated populations.

Growth Factor.	Tissue of Origin.
Interleukin I (IL-1) Interleukin 3 (IL-3)	Monocytes. Monocytes, Periosteum.
Epidermal Growth Factor (EGF)	Salivary Gland, Kidney, Mammary Gland, Pancreas, Duodenum, Others.
Platelet Derived Growth Factor (PDGF)	Osteoblast? Endothelial Cell? Platelet?
Fibroblast Growth Factors (acidic FGF) (Basic FGF)	Osteoblast, Endothelial Cell? Systemic Circulation?
Insulin-Like Growth Factor-I (IGF-I, Somatomedin C) Insulin Like Growth Factor-II (IGF-II)	Osteoblast, Fibroblast, Systemic Circulation Osteoblast, Fibroblast.
Transforming Growth Factor- β (TGF- β)	Osteoblasts, Platelets.
Osteoinductive Factors (OIF)	Osteoblasts? Epithelial Cells?
Bone Morphogenetic Proteins 1 to 7 (BMP-7)	Periosteal Cells. A range of other tissues (see Wozney, 1990)

Fig 1.3. Table adapted from Canalis et al, 1989 to list selected growth factors which have been studied because of their effect on bone cell proliferation, bone matrix synthesis and resorption and growth of bone cell precursors in vitro (CFU-f) together with their tissue of origin.

1.5c: MONOCLONAL ANTIBODY MARKERS.

An alternative strategy aimed at characterizing the differentiated osteoblast population has been to use isolated osteoblast-like cells as antigens to raise specific monoclonal antibodies. Nijweide and Mulder (1986) and Nijweide et al (1988) have reported a range of antibodies directed against foetal chick or foetal rat osteoblast-derived cells. One in particular, cOB7.3, reacted specifically with chick osteocytes (Bruder and Caplan, 1989; Nijweide and Mulder, 1986). Three antibodies directed against chick cells have been reported (Bruder and Caplan, 1989) of which one, SB-1, was subsequently found to be directed against an alkaline phosphatase epitope (Bruder and Caplan, 1990a) and two, SB-2 and SB-3, were directed against cells of the osteoblast layer but not osteocytes. Subsequently another antibody, SB-5, recognized chick osteocyte epitopes (Bruder and Caplan, 1990b). Using these antibodies it has been proposed that osteoblast differentiation comprises a series of discrete stages from an osteoprogenitor to the osteocyte, each characterized by a different surface antigen profile (Fig 1.4). To date there are no specific markers for osteogenic precursors.

Cell	Monoclonal Antibody
Osteo-progenitor	No probes currently available
Pre-osteoblast	SB-1 and AP +ve
Osteoblast	SB-1, SB-2, SB-3 and AP +ve
Osteocyte	SB-5 +ve OB 7.3 +ve AP -ve

Fig 1.4. Summary of antibodies expressed by embryonic chick osteoblasts at discrete stages of the osteoblast lineage (adapted from Bruder and Caplan, 1989; 1990b; Nijweide and Mulder, 1986). AP is alkaline phosphatase.

1.5d: OSTEOBLAST-DERIVED MATRIX PROTEINS.

The principal function of the osteoblast is the synthesis and secretion of the organic and mineral phases of the bone matrix. The organic matrix of bone is approximately 90% collagen and 10% non-collagenous proteins, a heterogeneous group of more than 200 proteins (Delmas et al, 1984) of bone cell derived (endogenous) origin, or non bone cell derived (exogenous) origin. Included with the endogenous, non-collagenous proteins of bone are a number of molecules which are either bone matrix specific or restricted in their distribution with much higher levels in the bone matrix than other tissues. As the non-collagenous proteins of bone have been reviewed extensively elsewhere (Termine, 1988; Robey, 1989; Triffit, 1987), only those of relevance as bone cell specific differentiation markers will be discussed.

Bone Collagens.

The main collagen types present in bone are the fibrillar collagen types I and V (Eyre et al,1990). Both are ubiquitous, being widely distributed throughout the body (Gage et al,1989), however, they are not found in hyaline cartilage. The hyaline cartilage matrix is more complex, characterized by the cartilage specific collagen types II, IX and XI together with small amounts of the widely distributed type IV collagen (Eyre et al, 1990). Despite the widespread distribution of collagen type I, expression of types I or II, either at a protein or mRNA level may be useful in distinguishing differentiation in an osteogenic or cartilaginous direction.

Non-Collagenous Proteins of Bone.

Osteopontin.

Osteopontin, also known as bone sialoprotein I or 2ar, a 44KDa glycoprotein rich in sialic acid is thought to be a vitronectin like attachment protein containing an Arg-Gly-Asp (RGD) attachment sequence (Butler,1989). It is produced by bone cells in vivo and in vitro, including rat calvarial cells (Franzen and Heidegard, 1985) and rat osteosarcoma derived ROS 17/2.8 cells (Prince and Butler, 1987). Polyclonal antisera raised against rat long bone osteopontin have been used to localize the protein to osteoblasts, osteocytes and fibroblasts close to sites of active osteogenesis (Mark et

al,1987a;1987b;1988). This was interpreted to mean that osteopontin was synthesized by osteoblasts, osteocytes and their immediate precursors. Weinreb et al (1990) studied Osteopontin, osteocalcin and alkaline phosphatase mRNA expression in sections of rat long bones by in situ hybridization. Osteopontin and osteocalcin were expressed in cells adjacent to the bone whilst alkaline phosphatase message was present in a large number of bone forming cells including osteoblast precursors that were many layers removed from the bone surface. These results suggest that osteopontin is of value as a late marker of osteogenic differentiation.

Synthesis of osteopontin by non-rodent osteoblasts has yet to be documented although there is unpublished evidence suggesting that it is not a major secretory product of normal adult human bone cells (Robey, 1989), and this may reflect a significant species difference. Although osteopontin is not unique to bone tissue, it is not widely distributed and is restricted to hypertrophic cartilage, renal and nervous tissue (Butler,1989) at both the protein and mRNA levels. It is identical to pp69, a phosphoprotein secreted by normal and transformed rat kidney cells, and the levels of protein were raised in 3T3 fibroblasts transformed with the H-ras oncogene (Craig et al, 1988). Osteopontin must, therefore, be used with caution as an osteoblast marker, particularly in vitro, where the possibility that a cell population may undergo spontaneous transformation cannot be ruled out.

Osteonectin.

Osteonectin is a relatively abundant non-collagenous protein produced by cells from a number of species including human, rabbit and bovine osteoblasts. There are distinct species differences, for example less osteonectin is incorporated into rat bones. The reason for this is unclear but may be related to the observation that in calves, the osteonectin compartment of woven bone is half that of lamellar bone (Cohn & Termine, 1985), and rat bones remain woven in character until they reach their adult weight, relatively later than in other species (Termine, 1988).

Although osteonectin levels in bone are over 1000 times greater than other tissues it is widely distributed and has been studied both at the protein and mRNA levels. In situ hybridization studies on foetal and adult mice (Holland et al, 1987) and human foetal tissue (Metsaranta et al, 1989), localized high levels of osteonectin mRNA to odontoblasts, osteoblasts, periosteal cells and hypertrophic cartilage cells. Lower levels of mRNA were found in osteocytes, skin, tendon, connective tissue surrounding the nerve trunk and the decidua of the placenta (Robey, 1989). Osteonectin has been detected immunocytochemically in osteoblasts and bone marrow cells (Bianco et al, 1985; 1988a; 1989; Jundt et al, 1987), odontoblasts (Tung et al, 1985) and periodontal ligament cells and some chondrocytes particularly hypertrophic chondrocytes (Bianco et al, 1988a; Jundt et al, 1987).

It is an acidic glycoprotein rich in cysteine with an Mr of 80KDa, virtually identical to a secreted protein produced by teratocarcinoma cells - SPARC¹ (Mason et al, 1986), or BM40 from the murine ELH (Englebreth-Holm-Swarm) basement membrane tumour (Dziadeck & Paulson, 1986). It has also been described as a culture shock protein in endothelial cells (Sage et al, 1984) and appears to be transiently activated in non bone tissues during periods of active growth or remodelling (Termine, 1988). It is also present continuously at low levels in tissues such as the renal distal tubule or salivary duct (Bianco et al, 1989) and it may have a role in Ca²⁺ transport and mineralization.

Whilst high levels of osteonectin are indicative of osteoblast activity in vivo, raised osteonectin levels in vitro may reflect local culture conditions rather than differentiation in an osteogenic direction making it of limited value as an in vitro marker of osteogenesis.

Osteocalcin and Matrix Gla Protein.

The two vitamin K dependent bone Gla proteins, osteocalcin and matrix Gla protein, characterized by the presence of γ -carboxyglutamic acid residues, are of considerable interest. Osteocalcin, a soluble 5700 M_r protein, is the only known protein specific to calcified tissues (Haushka, 1986; Robey, 1989; Price, 1983). Matrix Gla protein mRNA is seen in bone, cartilage, lung, liver and kidney, but the levels of protein

¹Secreted Protein Rich in Cysteine

found in bone are up to 50 times higher than in other tissues (Frazer and Price, 1988).

Specific antisera to osteocalcin have been used to determine its tissue distribution confirming that it is specific to calcified tissues and seen in osteoblasts and odontoblasts (Bronkers et al, 1985; 1989; Camarda et al, 1987). It appears in the matrix just before the onset of mineralization.

Bronkers et al (1987), studying histological sections of a rat tooth and long bone showed that differentiating osteoblasts and odontoblasts had histochemical alkaline phosphatase activity before the appearance of osteocalcin. Subsequently this pattern was seen when using in situ hybridization to localize osteocalcin and alkaline phosphatase message (Weinreb et al, 1990).

In vitro, osteocalcin is synthesised by osteoblast-like cells from a range of species (Beresford et al, 1984; Gershenfeld et al, 1987). Some osteosarcoma derived cell lines including the ROS 17/2.8 line synthesize osteocalcin whilst others such as the UMR 106 line do not (Price and Baukol, 1980; Frazer et al, 1988; Kaplan et al, 1985). In vitro, physiological concentrations of $1.25 (\text{OH})_2$ vitamin D_3 (calcitriol) stimulate osteocalcin production at the transcriptional level (Price and Baukol, 1980). Because of the small, soluble nature of the molecule the addition of monensin has been required to prevent diffusion from the cell prior to immuno-localization.

The function of osteocalcin remains unknown. Its synthesis is stimulated by $1.25(\text{OH})_2$ Vitamin D_3 which suppresses collagen synthesis in rat calvarial cultures (Rowe and Kream, 1982), alkaline phosphatase expression in osteosarcoma derived cell lines (Rodan and Rodan, 1984) and which is widely regarded as a resorptive stimulus (Raisz, 1972). Furthermore, bone particles harvested from warfarin treated animals were low in osteocalcin and were not efficiently resorbed by a population enriched for osteoclast-like cells (Lian *et al*, 1986). It has been speculated, therefore, that osteocalcin may have a role in resorption, perhaps in the coupling of osteosynthesis to osteolysis, rather than in bone formation (Robey, 1989).

Bone Proteoglycans.

The major proteoglycans present in the bone of most species are the chondroitin sulphate proteoglycans PG I (Decorin) and PG II (Biglycan) (Robey, 1989). They are not unique to bone and are seen in a diverse range of connective tissues. They are, therefore, likely to be of little use as markers of the osteoblast phenotype.

Rabbits are unique amongst mammals in expressing a small, bone specific proteoglycan, the keratan sulphate proteoglycan. The core protein with a M_r of approximately 80,000 shares homology with the human and bovine bone sialoprotein or BSP (Kinne and Fisher, 1987). A bone specific antibody has been raised against the keratan sulphate core protein which does not cross react with BSP from other species (Joyner *et al*, 1989; 1991),

and has been used as a late stage marker of osteoblastic differentiation.

1.6: CARTILAGE.

Three types of cartilage are recognized, hyaline, elastic and fibrocartilage. Hyaline cartilage is found in the epiphyseal growth plates, lining the articular surfaces of synovial joints and forming the cartilaginous core of developing bones. In fracture healing (Ham and Cormack, 1979) or ectopic ossification (Connor, 1983) hyaline cartilage precedes mineralization. This discussion, therefore, is directed towards hyaline cartilage. Chondrocytes synthesize metachromatic staining cartilaginous matrix and come to lie in lacunae within it. Typical chondrocytes are ovoid cells $<30\mu\text{m}$ in diameter. Each cell has a scalloped surface with occasional cytoplasmic processes extending into the matrix. Cytoplasmic organelles such as a granular endoplasmic reticulum or golgi apparatus are seen in young chondrocytes , but these are lost as the cell gets older. In addition, the number of cells present decreases with age.

Cartilage matrix consists predominantly of proteoglycans with keratan sulphate or chondroitin sulphate side chains, however, these molecules are found in a range of connective tissues and are not specific to cartilage. Similarly, the matrix proteins osteonectin and osteopontin are expressed in cartilage , bone and other tissues. Like bone, collagen is a major organic

component of the matrix: Types II, IV, VI, IX, X and XI are expressed and types II and IX are regarded as cartilage specific (Gage et al, 1989; Eyre et al, 1990).

1.7: CHONDROID BONE.

Type I collagen is not a feature of cartilage and has been used to distinguish cartilage from bone. In certain situations, for example the rat penile bone or mandibular condyle (Beresford, 1981), small areas of large spherical or ovoid cells resembling chondrocytes are surrounded by a mineralized matrix resembling bone and co-expressing collagen types I and II. This has been termed chondroid bone. Chondroid bone is an important feature of in vivo and in vitro models of osteogenesis.

1.8: FIBROBLASTS.

Fibroblasts are a cellular component of all of connective tissues including periosteum, endosteum and marrow. In vivo they have a characteristic spindle shaped appearance. They are metabolically active, producing and maintaining the extra cellular matrix including collagen, mainly types I and III, glycosaminoglycans and elastin. In-vitro, the term 'fibroblastic' or 'fibroblastoid' has been applied to cells with a range of morphologies from spindle shaped or stellate to large flattened cells extending cellular processes out over the base of the culture flask and characterized by intra

cellular filamentous structures termed stress fibres. It is unclear, however, how they relate to fibroblasts in vivo. On histological grounds a variety of cell types have been grouped with the fibroblast (Ham and Cormack, 1979) including the reticular cell or the myofibroblast, however, there are no widely accepted markers of these cell types and the hierarchical relationship between them remains unclear.

In vitro studies have shown that cells with a fibroblastic morphology are precursors of a number of cell types. Adipocyte precursors, for example, are morphologically fibroblasts and synthesize collagen types I and III. On differentiation there is a switch in favour of type IV production, active secretion of a basement membrane and lipid accumulation (Green and Meuth, 1974; Weiner et al, 1989). Calvarial or marrow derived stromal cells appear fibroblastic in vitro, and form a calcifying nodules resembling bone or cartilage if allowed to grow to confluence under certain conditions (Bellows et al, 1986; Maniatopoulos et al, 1988; Beresford et al, 1991) or a bone and cartilage like tissue capable of supporting haemopoiesis in vivo (Friedenstein, 1980; Patt and Maloney, 1982). Confluent cultures derived from pluripotential stromal cell lines have been described, capable of forming a range of differentiated phenotypes in vitro including adipocytes, multinucleate cells resembling muscle and nodules resembling bone or cartilage, whilst in subconfluent culture they appeared fibroblastic (Grigoriadis et al, 1988). Cells appearing fibroblastic in vitro, therefore, may reflect a

relatively undifferentiated mesenchymal precursor population rather than a distinct phenotype.

There are clear differences between fibroblastic populations in vitro derived from different anatomical sites. Bayreuther et al (1988; 1989) addressed the question of whether there is a differentiated fibroblast by studying human skin fibroblasts. On the basis of morphology, growth potential and the cell protein profile analyzed by two dimensional gel electrophoresis, he proposed that a fibroblast lineage could be defined, ranging from small, spindle shaped pre-mitotic cells to large flattened post-mitotic differentiated cells with characteristic stress fibres. It is not known whether a similar lineage for marrow or periosteal fibroblasts is present in the marrow stromal system. Some evidence in support of an analogous lineage comes from adherent marrow populations derived from Vervet Green Monkeys, where large flattened cells had a lower growth potential to smaller spindle shaped cells (Kramvis and Garnett, 1987).

The cytoskeletal proteins, vimentin and α smooth muscle actin have been demonstrated immunocytochemically in adherent marrow stromal cultures localized to stress fibres (Charbord et al, 1990). In vivo, these proteins are features of myofibroblasts (Gown, 1990). In studies on wound healing in vivo they are expressed transiently and probably indicate fibroblastic differentiation or plasticity in the fibroblastic phenotype (Darby et al, 1990). Although stromal fibroblasts in vitro

may represent a population comprising relatively undifferentiated mesenchyme cells the possibility remains that the myofibroblast may be a differentiated fibroblastic phenotype which has yet to be fully characterized.

1.9: ADIPOCYTES.

1.9a: EXTRA-MEDULLARY ADIPOCYTES.

Fat tissue has been described as a loose association of adipocytes characterized by dense cytoplasmic accumulations of lipid and stromal vascular cells held in a collagenous matrix (Johnson & Greenwood, 1988). Both marrow and extra medullary fat will be considered. Two types are considered, brown fat which is vascular, richly innervated and associated with the physiological response to cold stress, and white adipose tissue which is less vascular, less well innervated with an insulation and storage role. Extra-medullary fat has been studied extensively in vivo and in vitro in culture using rodent pre-adipocyte cell lines (Green and Kehinde 1975; 1976; Negrel et al, 1970; listed by Ailhaud et al, 1982).

Undifferentiated cells, capable of rapid growth have a fibroblastic morphology. Early events in the conversion to mature adipocytes are triggered by the cessation of growth and the emergence of a specific set of phenotypes at the morphological, mRNA and protein levels before intracellular accumulation of lipid is observed (Ailhaud et al, 1982;

1987). In studies on the 3T3-L1 pre-adipocyte cell line (Filpack et al, 1989; Hoerl and Scott, 1989) a pre-differentiation growth arrest state has been described when cell proliferation is restricted and cells begin to acquire the granular cytoplasm regarded as characteristic of a pre-adipocytic state (Roncari and Van, 1978; Van and Roncari, 1982; Cormack, 1989). This progressed to a non-terminally differentiated state in which cells began to assume features of differentiated adipocytes whilst retaining a limited capacity for growth. Terminally differentiated cells were incapable of further growth and expressed the adipocyte phenotype. Consistent observations have been made on the Ob17 clonal cell line (Ailhaud et al, 1989). Growth arrest preceded 'early' events in differentiation leading to cells with a limited growth potential whilst expressing some features of differentiated adipocytes at a morphological, protein and mRNA level. 'Late' events lead to the loss of proliferative potential and acquisition of the adipocytic phenotype. These observations are summarized in Fig 1.5.

Following growth arrest, cells become larger, rounded and begin to accumulate small cytoplasmic lipid droplets giving a granular appearance widely regarded as indicative of a pre-adipocytic state (Cormack, 1989; Roncari and Van, 1978; Van and Roncari, 1982). At a protein level, early markers include a large set of enzymes associated with triglyceride synthesis such as lipoprotein lipase (LPL), fatty acid synthetase, phosphatidic acid phosphatase, glycerol-3-phosphate

dehydrogenase (GPDH) and acetyl CoA carboxylase (Filpack et al, 1989). Up regulation of differentiation specific mRNA's coding unknown proteins have been described. These include pOb24 or pGH3 (Ailhaud et al, 1987). Terminally differentiated cells are characterized by increasingly dense cytoplasmic accumulations of lipid leading to large cytoplasmic lipid filled vacuoles which take up Oil-Red-O or sudan black stain, and a marked increase in LPL or GPDH activity (Filpack et al, 1989; Krawisz and Scott, 1982). More recently, specific protein markers have been described associated with late events in adipose conversion. Examples include a 0.7Kb cDNA, PAL422, coding a 14KDa intra-cellular fatty acid binding protein AP2 (subsequently referred to as PAL422(AP2)) (Lane et al, 1990; Bernlohr et al, 1984), and pAd20, a 1Kb cDNA coding a 28KDa protein, adipsin, with putative protease activity (Spiegelman et al, 1983).

Fig 1.5 Table compounded from observations on cloned rodent pre-adipocyte cell lines illustrating key early and late events in adipocytic differentiation.

**UNDIFFERENTIATED
ADIPOCYTE PRECURSORS.**

Rapid growth state.

Undifferentiated cells have a fibroblastic appearance.

**EARLY EVENTS IN ADIPOCYTE
DIFFERENTIATION.**

Pre-differentiation growth arrest state.

Morphology.

Cells become larger, rounded and begin to accumulate small cytoplasmic lipid droplets giving a granular appearance.

Induction of enzymes associated with fatty acid metabolism.

e.g: Lipoprotein lipase (LPL),
Glycerol 3 phosphate
dehydrogenase (GPDH).

Transcription of differentiation specific mRNA's coding unknown proteins.

e.g: pOb24,
pGH3.

**LATE EVENTS IN ADIPOCYTE
DIFFERENTIATION.**

Terminal differentiation state.

Transcription and synthesis of adipocyte differentiation specific proteins.

These include;
pAL 422 mRNA coding AP2 protein
pAd 20 mRNA coding adipsin.

1.9b: MARROW FAT.

In vitro marrow and extra-medullary fat have a similar morphological appearance, nevertheless, there are distinct

differences in terms of physiological role and cellular responsiveness to specific biochemical agents. In acute starvation, lipolysis occurs in extra medullary adipocytes whilst marrow fat remains unaltered (Tavassoli, 1974). Medullary adipocytes, however, are sensitive to changes in the marrow micro environment. In osteoporosis, trabecular bone appears to be replaced by fatty marrow (Burkhardt et al, 1987) (chapter 7) and there is a reciprocal relationship between the amount of haemopoietic and adipocytic marrow present in myeloproliferative disorders (Hirata et al, 1989). At a biochemical level, insulin promotes glucose uptake and lipogenesis in extra medullary fat but not in marrow adipocytes whilst adipocytic conversion in the latter group could be induced by hydrocortisone (Greenburger, 1978; 1979). Expression of adipsin and PAL422(AP2) has been demonstrated during adipogenic conversion of the murine preadipocyte cell line BMS2 (Pietrangeli, et al, 1988; Gimble et al, 1989; 1990). Alkaline phosphatase activity has been observed in adipogenic human bone marrow cultures in vitro (Bianco et al, 1988). It has been shown in this thesis using a rat marrow model that PAL422(AP2) can be used as a marker for marrow adipocytes. By modulating the culture conditions in favour of expression of the osteoblastic or adipocytic phenotype it was possible to up regulate osteocalcin or PAL422(AP2) mRNA, thus demonstrating the presence of both osteogenic and adipocytic markers within the same system.² In a preliminary report, Rosen et al (1990)

²Beresford J. N., Leboy P. S., Deviln C., Bennett J. H., Owen M. E., - work currently in progress,

demonstrated that TGF- β or osteoinductive factor (OIF), inducers of osteoblastic differentiation, promote alkaline phosphatase expression in BMS2 cells and they are currently looking at the expression of other markers of the osteoblast phenotype in this cell line.

Differentiation in extra medullary fat remains one of the best documented examples of differentiation from stromal-vascular precursors. Phenotype specific differentiation markers have been identified which are potentially useful in the study of marrow adipocytes and hence of osteogenic marrow stromal populations.

1.10: IN VITRO MODELS FOR THE STUDY OF OSTEOLAST-LIKE CELLS.

Progress in bone cell biology has been greatly facilitated by the development of tissue culture techniques for the isolation and growth of cell populations with osteogenic potential which can be studied in vitro. These include primary stromal populations derived from bone marrow or periosteum, non-transformed cloned cell lines and osteosarcoma derived cell lines.

1.10a: ISOLATED CELL POPULATIONS WITH OSTEOGENIC POTENTIAL.

Cell populations used within a short time of being obtained from the in vivo state are more likely to be representative of their counterparts in vivo. Furthermore, it is reasonable to assume

that freshly isolated cells from a healthy animal are a non-transformed population. Examples of cells obtained in this way which have been used for the study of osteoblast lineage relationships are as follows:

- i) collagenase digests of calvarial cells
- ii) adherent marrow stromal populations
- iii) bone explant cultures.

Collagenase Digests of Calvarial Cells.

In the first, originally described by Peck et al (1964), foetal rat calvarial bones were cleaned of superficial dura and periosteum, then placed in crude collagenase extracts for up to 90 minutes. When cells released by collagenase digestion were collected sequentially at regular time intervals, distinct populations were isolated. Early released cells were thought to be those lying superficially on the calvarial surface with features of an undifferentiated mesenchymal population including production of collagen types I and III. Later released cells represented a more differentiated population, producing less collagen type III, and were considered to be osteoblast-like on the basis of their cyclic AMP response to PTH and alkaline phosphatase expression (Wong and Cohn, 1974; 1975; Luben et al, 1976; reviewed by Rodan and Rodan, 1984). Subsequently, these late released osteoblast-like populations prepared from collagenase digestion of foetal or neonatal rat or mouse calvaria have become widely known as 'isolated osteoblast-like populations'.

When tested in vivo in diffusion chambers, the earlier released populations were less able to form bone than the isolated osteoblast-like cells (Simmons et al, 1982). Bellows et al (1986) have demonstrated that isolated osteoblast-like populations form mineralizing nodules resembling bone in vitro, whilst early released cells do not (section 1.14).

Adherent Marrow Stromal Populations.

When a single cell suspension of marrow cells is plated into culture flasks in vitro, stromal cells rapidly attach to the tissue culture surface and proliferate to form an attached stromal layer whilst the majority of haemopoietic cells remain in the supernatant. The stromal layer is a heterogeneous group of cell types including stromal and endothelial elements as well as cells of the macrophage-monocyte series, and it contains those stromal elements necessary for the growth and maintenance of haemopoietic stem cells in vitro (Dexter et al, 1977).

Bone Explant Cultures.

This technique was described for the culture of human bone cells (Beresford et al, 1983) and is ideally suited to situations where only small samples of bone are available. Bone fragments were scraped free of soft tissue then placed in culture. After about 21 days extensive cellular outgrowths could be seen growing from the cultures and the cells could be isolated, harvested and replated. Cells from explant cultures were

responsive to parathyroid hormone, and produced osteocalcin on stimulation with $1.25(\text{OH})_2$ Vitamin D_3 (Beresford et al, 1984).

1.10b: CLONED OSTEOBLAST-LIKE CELL LINES.

Although the use of cell populations obtained from the three sources described above has provided a large amount of valuable information on osteogenesis in culture, their main drawback is heterogeneity as they contain osteogenic cells at varying stages of differentiation and cells from other tissues. To overcome this problem, attempts have been made to isolate cloned cell populations from all three sources. The greatest number of studies using this approach have been made using the later calvarial digests.

The MC3T3-E1 line, derived from neonatal mouse (Kodama et al, 1981; Kumegawa et al, 1984; Sudo et al, 1983), and cloned cell lines derived from foetal rat (Aubin et al, 1982), isolated by limiting dilution, have osteoblast-like characteristics. Both respond to PTH with an increase in intra-cellular cAMP, form collagen type I and produce nodules of a mineralizing bone-like matrix in vitro. In the former, one cell line was isolated, whilst in the latter, several clones were compared. Although each clone had features of the osteoblastic phenotype, they varied with respect to alkaline phosphatase, parathyroid hormone and PGE_2 responsiveness and no two clones were identical. This is consistent with current views on osteoblast lineage (section 1.3), in which each osteoblastic precursor is thought to differ,

representing a marginally different stage in the lineage. With repeated passaging, however, rat calvarial clones were not phenotypically stable (Aubin et al, 1988) and lost their hormone responsiveness.

More recently, murine IgM monoclonal antibodies directed against rat marrow fibroblastoid cells (Sullivan et al, 1989) were used in a protocol involving complement mediated cell lysis to give a population enriched in osteoblast-like cells. From this, limiting dilutions were used to isolate two clonal populations with increased alkaline phosphatase and PTH responsiveness, capable of forming mineralized nodules in vitro (Bernier et al, 1990). In the presence of $1.25(\text{OH})_2$ vitamin D_3 , neither population expressed osteocalcin at the protein or mRNA level.

An alternative approach was to use a combination of TGF β and EGF to reversibly transform single cell suspensions of osteoblast-like cells in soft agarose culture (Guenter et al, 1989). Discrete, progressively growing colonies formed which were subsequently isolated, subcultured and characterised. Again, clonal populations differed in alkaline phosphatase activity, PTH responsiveness and expression of osteocalcin mRNA. One clone was continuously subcultured for 22 passages to assess the stability of markers of the osteoblast phenotype in vitro. These remained stable initially but, by the 13th passage, were lost. If clonal populations of osteoblast-like cells are to be studied, therefore, it is necessary to design experiments on cell populations as close as possible to primary culture.

Attempts have been made to obtain clones of osteoblast-like cells by exposure to an immortalizing vector containing SV40 viral DNA. One clone, RCT3, was alkaline phosphatase rich, increased cAMP production in response to PTH and formed a bone-like matrix in diffusion chambers. Whilst these features remained stable over 30 passages, RCT3 cells were tumorigenic on subcutaneous injection into athymic mice. It remains uncertain whether these lines represent genuine characteristics rather than transformation or immortalization dependent changes (Rodan et al, 1988). Human osteoblast-like cells derived from trabecular explant culture have been transfected with SV40 DNA and reported to give immortalized cell lines with increased AP activity, producing osteocalcin and mineralizing nodules in vitro (Williams et al, 1990). Data is not yet available relating to the stability or tumorigenicity of these cultures. Mouse teratocarcinoma cells have similarly been immortalized with SV40 to give a cell line which can form a mineralized matrix in vivo and in vitro (Kellermann et al, 1990).

A cloned osteogenic cell line has been obtained from murine marrow (Benayahu et al, 1989) which formed bone on in vivo assay in sealed diffusion chambers.

1.10c: OSTEOSARCOMA-DERIVED CELL LINES.

Two cell lines have been used extensively to study bone metabolism in vitro. These include the ROS 17/2 line, in particular the ROS 17/2.8 subclone derived from a spontaneous

rat osteosarcoma (Majeska et al, 1980) and UMR 106, a rat line derived from a radioisotope induced osteosarcoma (Partridge, 1981). Both show an enhanced alkaline phosphatase responsiveness and respond to PTH with an increase in cAMP production. Both synthesize Gla proteins. UMR 106 cells synthesize matrix Gla protein but not osteocalcin whilst ROS 17/2 cells readily form osteocalcin but did not readily synthesize matrix Gla protein (Frazer and Price, 1990; Frazer et al, 1988; Price and Baukol, 1980). These observations may reflect the transformed nature of the cell lines. Alternatively, they may mean that osteocalcin and matrix Gla protein are synthesized by different subsets of bone cells and are of value as phenotypic markers for different osteoblastic populations.

As with non-transformed cells, phenotypic changes have been reported in these lines when kept in long term culture, including loss of hormone responsiveness and alterations in collagen expression (Gutierrez et al, 1986; Grigoriadis et al, 1985).

One reason for the usefulness of these cell lines is the potential for obtaining unlimited numbers of cells for biochemical investigations. The fact that they are transformed and show some phenotypic instability means that they are unsuited to the study of hierarchical relationships in stromal tissues.

1.11: EVIDENCE IN SUPPORT OF THE STROMAL HYPOTHESIS.

1.11a: EARLY WORK.

The close relationship between fibrous connective tissue and bone has been appreciated for many years, indeed, organ culture studies in the 1930's showed that bone could develop in vitro from periosteum derived from embryo chick long bones (Fell,1932). Furthermore, other tissues not destined to produce bone do not ossify under the same conditions suggesting some degree of specificity and differentiation in the periosteal organ cultures. This did not imply, however, that osteogenic potential was a unique feature of the fibrous connective tissue lining bone, merely that the specific biochemical environment in the organ culture favoured osteogenic differentiation (Murray,1936). Given a suitable biochemical environment stromal cells from a range of tissues which were not normally osteogenic could form bone. Experimental ossification, for example, had been reported in the kidneys following ligation of the renal vessels (Sacerdotti and Frattin, 1902; Bridges and Pritchard, 1958). When fragments of canine bladder epithelium were transplanted to ectopic sites such as muscle tissue or subcutaneous fat, bone was formed. However, osteogenesis could not be induced on implantation into the parenchyma of kidney, liver, spleen or lung (Huggins,1931; Huggins and Sammett, 1933; Huggins, 1968).

1.11b: EVIDENCE FROM STUDIES ON MARROW REGENERATION.

Studies of marrow regeneration received considerable attention in the years following World War II with the development of radiobiology, and were initially designed to investigate the haemopoietic compartment. Detailed light microscopic studies on tissue regeneration following depopulation of the marrow cavity by surgical ablation (Branemark et al, 1969; Maloney and Patt, 1969; Meyer-Haume et al, 1971; Patt and Maloney, 1975) or perfusion facilitated description of a sequential series of events mirroring a developmental process leading to the re-establishment of a functional marrow (Fig 1.6). Following ablation a blood clot fills the cavity to be followed by neovascularization, proliferation of a fibrous connective tissue from the bony surfaces and osteoblast differentiation. Finally, following formation of bone trabeculae and the reconstruction of a sinusoidal microcirculation a haemopoietic marrow forms. From these studies it appeared that the development of a haemopoietic marrow required a distinct supporting stroma, consisting of a number of cell types such as the fibroblast, the osteoblast or the adipocyte which share common features.

Fig 1.6 Approximate sequence of events during tissue regeneration following ablation of the marrow cavity (from Owen, 1980).

Time after ablation (days).	Events in the marrow cavity.
0	Removal of marrow from cavity.
0-2	Blood clot fills depleted region of cavity.
2-7	Proliferation of connective tissue from bone surfaces.
7-10	Differentiation of cells into osteoblasts. Formation of bone trabeculae. Reconstruction of sinusoidal microcirculation. Arrival of haemopoietic stem cells.
10-30	Resorption of trabecular bone. Haemopoietic marrow re-established.

1.11c: EVIDENCE FROM STUDIES ON THE HAEMOPOIETIC LINEAGE.

When lethally irradiated mice were injected with marrow from a healthy syngeneic animal, the marrow was repopulated and it recovered. Autopsy on experimental animals at different stages of recovery demonstrated discrete nodules projecting from the surface of the spleen. Histological studies showed that these nodules were composed of cells of haemopoietic origin, in particular cells of the erythrocyte or granulocyte lineages (Till and McCulloch, 1961). Using chromosomal markers it was demonstrated that these clones were of donor origin and

that each cell was the product of a single CFU-S or colony forming unit-spleen (Becker et al, 1963; Abramson et al, 1977).

Subsequently, the spleen colony technique was used as an assay for CFU-S, and it was demonstrated that a single pluripotent CFU-S could repopulate the haemopoietic compartment of an irradiated recipient (Becker et al, 1963; Abramson et al, 1977). Specific retroviral markers have been introduced into donor CFU-S and used to repopulate the haemopoietic compartment of irradiated syngeneic W/W^v mice. Analysis of DNA from the spleen, bone marrow and thymus of the animals confirmed that a single CFU-S had the potential to repopulate the host with a range of myeloid and lymphoid tissues (Dick et al, 1985; Lemnisha et al, 1986).

Whilst in vivo assays such as the spleen colony technique provided indirect information about the CFU-S, their relative insensitivity did not allow for the investigation of specific factors promoting the proliferation and differentiation of precursor cells to give the differentiated haemopoietic phenotypes (Fig 1.7). Initially, in vitro culture conditions for the growth of discrete haemopoietic colonies had to be defined. Next, the work was extended to allow the investigator to direct differentiation within colonies in vitro along a pre-selected pathway towards clonal populations enriched for a particular phenotype. Finally, the culture conditions had to be analyzed to establish the nature of specific factors

directing differentiation towards a particular phenotype (Metcalfe, 1984). From these studies it became apparent that much of the control of haemopoiesis is mediated by a group of glycoprotein molecules termed haemopoietic growth factors, acting at an autocrine or paracrine level, some of which have been isolated and cloned (Metcalfe, 1988; 1989).

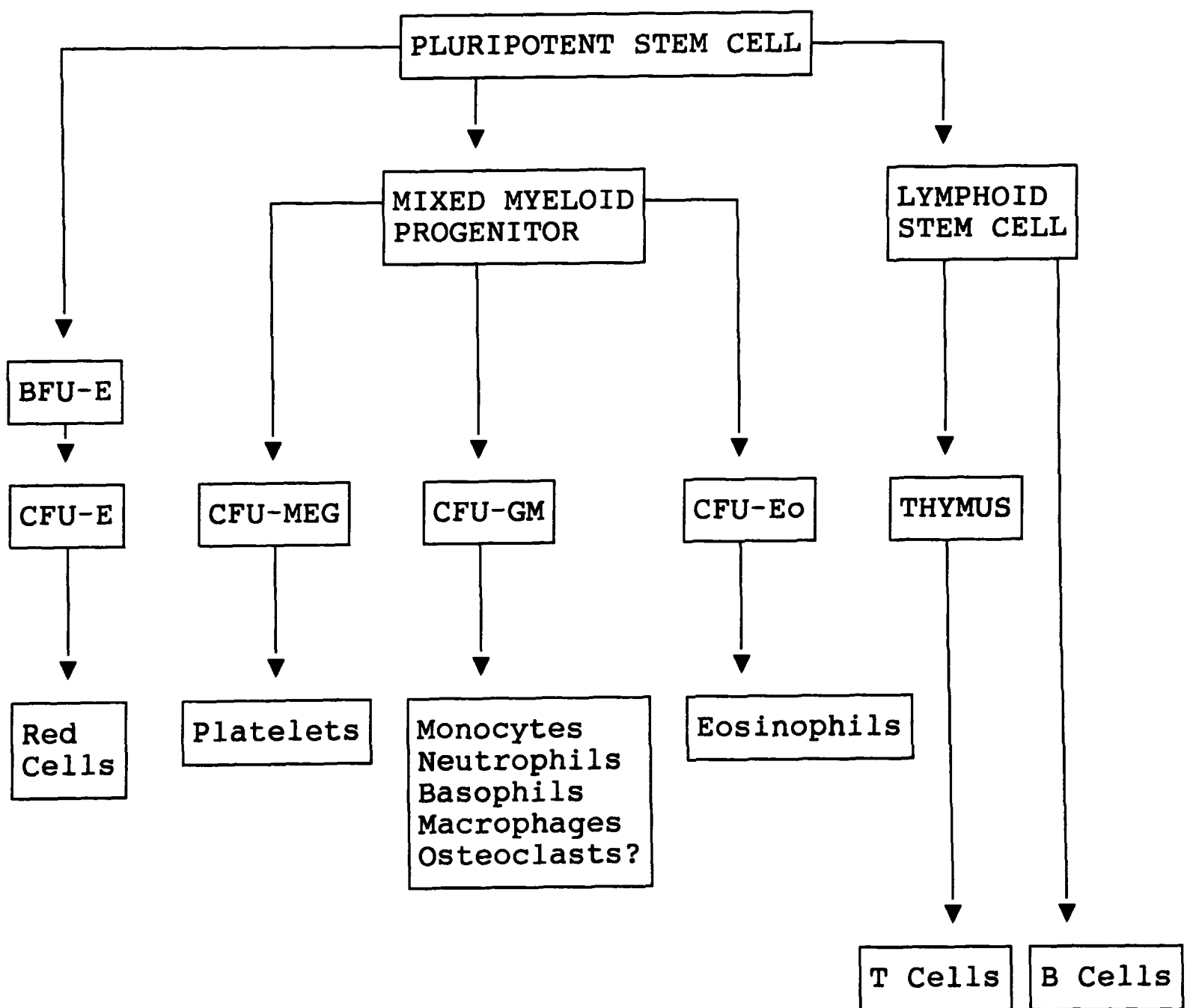


Fig 1.7. Diagrammatic representation of the haemopoietic lineage. CFU-E represents the erythrocyte precursors or colony forming units-erythroblastic; CFU-GM, the granulocyte/monocyte precursor; CFU-Eo, the eosinophil precursor; CFU-MEG, the megakaryocyte precursor, and the common pluripotential haemopoietic stem cell is the CFU-S.

It is now widely accepted that the CFU-S do not give rise to cell lines of the supporting marrow stroma. Evidence comes from studies in which different cell or antigenic markers were used to distinguish between tissues of host or donor origin. Stromal tissue from a radiation chimera, for example, is of host origin whilst haemopoietic cells are derived from that of the donor (reviewed by Friedenstein, 1976; Owen, 1980; 1985). Studies on patients with blood dyscrasias support the view that marrow stromal cells have a separate lineage to those of the haemopoietic system. Fibroblastic colonies from leukaemic patients in adherent marrow stromal culture were uniformly negative for the philadelphia chromosome (Lim and Izzaguere, 1986; Castro-Malaspina et al, 1982; Holling et al, 1984) and are probably not of leukaemic origin. In marrow transplant recipients CFU-F have been found to be of host origin (Friedenstein et al, 1978; Hollings et al, 1984; Simmons et al, 1987; Athanasou et al, 1990).

Some controversy remains. Keating et al, (1982) investigated the origin of the adherent marrow stromal layer in marrow graft recipients from immunologically identical donors of the opposite sex, and found, using the Y chromosome as a marker, that cells of the stromal layer may be of donor origin. Whilst this was interpreted to indicate a common precursor for haemopoietic and stromal tissue the adherent stromal layer is heterogeneous and may include other cell types such as endothelial cells or macrophages. Thus, the difference in results could be due to difficulty in accurately identifying

the cell populations studied (Dexter, 1982). Subsequently, adherent marrow cell cultures from normal and leukaemic patients were immortalized with the SV 40 virus (Singer et al, 1987) before being cloned. Individual clones had cell surface markers characteristic of the haemopoietic lineage whilst expressing proteins such as vimentin, α -smooth muscle actin and the collagens typical of a stromal phenotype. If these results can be repeated they may profoundly influence current opinion on stromal and haemopoietic ontogeny.

1.11d: EVIDENCE FROM STUDIES ON STROMAL TISSUES.

In Vivo Studies on Mixed Adherent Stromal Populations.

Marrow, implanted ectopically formed a bone-like tissue capable of supporting haemopoiesis (Freidenstien, 1968; 1973; 1974; Tavassoli and Crosby, 1968). On implantation in sealed diffusion chambers, marrow cells from a range of species including rat (Mardon et al; 1987), rabbit (Ashton et al, 1990; Bab et al, 1984), dog (Johnson et al, 1988), guinea pig (Freidenstien, 1976) and pig (Thompson et al, 1991) formed tissues resembling fibrous tissue, bone or cartilage on observation at both light and electron microscopic levels (Ashton et al, 1980; Bab et al, 1984). Subsequently, this resemblance was confirmed by immunocytochemical demonstration of extra cellular matrix macromolecules similar to those found in bone and cartilage (Ashhurst et al, 1990; Mardon, et al, 1987). These included types I and V collagen in bone with

types II and IX collagen in cartilage. Both chondroitin and keratan sulphate proteoglycans were found in bone and cartilage. In diffusion chambers containing rabbit derived cells a proteoglycan uniquely found in rabbit bone was identified (Joyner et al, 1989; 1991). Bone specific monoclonal antibodies raised against embryo chick tissue (Bruder and Caplan 1989; 1990a and b; - section 1.5c) recognize a bone-like tissue in diffusion chambers containing 5 day chick tibial marrow. Cells embedded within a collagen type I rich mineralized matrix stained particularly for SB-5, which uniquely recognizes the cell surface of osteocytes (Bruder et al, 1990c).

There is good evidence to suggest that tissue formed in vivo in diffusion chambers is derived from a small number of precursor cells. Sequential analysis of a series of chambers from day 0 to day 20 post-implantation has shown that initially, the number of cells within a chamber drops rapidly, to be repopulated by a tiny number of precursors (Bab et al, 1986). It was estimated that about 15 cells out of 107 whole marrow cells explanted into the chamber gave rise to all the cells present representing a number of differentiated phenotypes. Thus, it was concluded that these cells were characteristic of stem cells.

Repeated passaging of adherent marrow cell populations gave stromal populations capable of forming bone and cartilage like tissues on implantation in vivo in sealed diffusion chambers

(Friedenstein, 1976) and populations retained their osteogenic potential when tested after 17 passages. On ectopic transplantation using an open system intra-muscularly or under the renal capsule stromal cells formed a marrow organ including bone and adipose tissue capable of supporting haemopoiesis (Friedenstein et al, 1974; 1982; Patt et al, 1982). Using sex chromosomes and strain histocompatibility antigens to distinguish between tissue of donor and recipient origin Friedenstein et al (1978) were able to confirm that the ossicle was of donor origin whilst the associated haemopoietic tissue remained that of the host, thus the adherent marrow stromal population had osteogenic potential and the potential to provide a haemopoietic micro-environment. Furthermore, kinetic studies have demonstrated (Friedenstein, 1987), that osteogenic stem cells have the capacity to replicate under standard conditions in vitro.

Marrow stromal populations differ in their ability to support haemopoiesis. Adherent stromal populations from red or yellow marrow have an identical fibroblastic appearance in culture, yet form marrow organs on implantation in vivo resembling the appearance of the marrow from which the donor cells were originally derived (Patt et al, 1982). Interestingly, the one difference between first passage stromal cultures derived from either marrow type was the histochemical demonstration of α -naphthylbutyrate esterase (α -NBE), and the abundance of α -NBE in fibroblastic cultures from yellow marrow suggest that it

may be a useful marker of marrow pre-adipocytic tissue (Bainton et al, 1986).

Assay of Clonal Populations of Stromal Cells.

By plating a suspension of marrow cells at low density, discrete adherent stromal colonies form in primary marrow culture (Friedenstein, 1976; Owen, 1987), and each has a typical fibroblastic appearance. A diverse range of approaches including the use of chromosome markers, time lapse cinematography, sex karyotyping and the observation that the number of colonies formed is linearly related to the number of cells explanted, has provided strong circumstantial evidence that each was derived from a single cell, termed a CFU-F or colony forming unit-fibroblastic (Friedenstein, 1987). However, rigorous proof of the clonality of these populations must await the application of molecular biological techniques such as retroviral labelling to the marrow stromal system.

Colonies in comparable primary cultures were heterogeneous with respect to size, morphology and histochemical staining characteristics (Friedenstein, 1976; Owen et al, 1987). After two weeks in vitro colonies ranged from less than 50 to more than 10^4 cells. Also, the number of colonies varied with the density of cells explanted into the culture flask and the culture conditions. By comparing the number and morphology of colonies formed under different conditions this system has been used as an in vitro assay of CFU-F in both human and

animal models (Castro-Malaspina et al, 1980; 1982; Friedenstein, 1976; Owen et al, 1987). This type of approach may help to determine what factors promote the attachment and proliferation of the CFU-F, and to develop an in vitro model in which differentiation within a colony may be directed towards a pre-selected stromal phenotype.

Conventionally, the number of colonies formed per cell inoculum is described as the colony forming efficiency (CFE). In standard conditions using 20% serum, CFE falls with a decrease in explantation density of the marrow cells (Friedenstein, 1990a). In the presence of irradiated feeder cells CFE remained approximately constant and the relationship between the number of colonies and number of nucleated marrow cells explanted remained linear. From this it was possible to estimate that, in rabbits and guinea pigs, CFU-F occurred with a frequency of approximately $1:10^5$ nucleated marrow cells (Friedenstein, 1990b).

Differentiation within stromal colonies may be influenced by a range of factors. Hydrocortisone (Hc) enhanced AP activity in rabbit marrow stromal colonies (Owen et al, 1987), and fat accumulation in colonies derived from human marrow stroma (McIntyre and Bjornson, 1986). In murine marrow cultures in the presence of Hc and 20% horse serum, adipocytic and fibroblastic cells were observed within the same stromal colony, some of which expressed AP activity (Kodama et al, 1983). Conversely, epidermal growth factor suppressed AP

activity (Owen et al, 1987).

Stromal colonies grown in liquid culture synthesize collagen types I and III, but not factor VIII or laminin (Castro-Malaspina et al, 1980; Lim and Izzaguere, 1986). In a semisolid medium with hydrocortisone, human plasma and PHA-LCM³ colonies characterized by cells with lipid inclusions and expressing collagen type IV and laminin formed (Lim and Izzaguere, 1986). These colonies were composed of cells considered analogous to the reticular cells of the bone marrow stroma and derived from a putative 'reticulo-fibroblastoid' precursor or CFU-RF. The relationship between the CFU-F and the CFU-RF, however, remains unclear. Furthermore, the pattern of lipid inclusions, collagen type IV expression and synthesis of basement membrane protein is suggestive of adipocytic differentiation. The relationship of the CFU-RF to the adipocytic precursor has not been explored and it is not known if 'reticulofibroblastoid' cells express adipocytic differentiation markers in vitro.

The in vitro assay of CFU-F is in its infancy, even so it is possible to modulate phenotypic expression within stromal colonies by manipulating culture conditions, allowing conclusions to be drawn about the nature of the CFU-F. Further definition of serum free conditions promoting stromal growth, and of conditions allowing phenotypic expression at a colony

³phytohaemagglutinin stimulated, leucocyte conditioned medium.

level will be required if the in vitro assay of CFU-F is to be used to its full potential.

In Vivo assay of CFU-F.

After isolation and repeated passaging, larger stromal colonies were expanded to give a sufficient cell number for in vivo assay of their osteogenic potential in sealed diffusion chambers. 30% of those tested had the capacity to form bone (Friedenstein, 1987). Furthermore, colonies isolated and expanded in this way had the capacity to form a bone-like tissue which could support haemopoiesis in a 'marrow organ' when explanted ectopically under the renal capsule (Friedenstein, 1980). This marrow organ was characterized by a range of stromal tissues of donor origin; bone, cartilage, fat and fibrous tissue, whilst the haemopoietic cells were from the host.

This demonstration that a stromal precursor had the capacity both for extensive proliferation, and to give rise to a range of differentiated phenotypes, must remain one of the most compelling pieces of evidence in favour of a putative stromal stem cell.

1.12: EVIDENCE FROM CLONAL CELL LINES THAT CHONDROCYTES, OSTEOLASTS, ADIPOCYTES AND FIBROBLASTS SHARE A COMMON ORIGIN.

Clonal lines have been reported which give multiple stromal phenotypes in vitro. C3H10T $\frac{1}{2}$ CL8(10T $\frac{1}{2}$), a clonal mouse embryo derived fibroblast line (abbreviated to 10T $\frac{1}{2}$) differentiated in the presence of 5' azacytidine to give multinucleate muscle cells, adipocytes and chondrocytes (Taylor and Jones, 1979). Equivalent doses of 5' azacytidine caused malignant transformation in adult cells (Taylor and Jones, 1982), and it is not known if the observed changes in the presence of 5' azacytidine are a model of normal cellular differentiation or are secondary to malignant transformation (Taylor and Jones, 1982). Similar results have been reported for swiss mouse 3T3 fibroblasts (Taylor and Jones, 1979). 3T3 fibroblasts, however, cannot be regarded as strictly clonal as they are a population isolated by repeated passaging on the assumption that a clonal line would emerge, whilst other cells present would not survive (Todaro and Green, 1963). Subsequently, 3T3 fibroblasts were subcloned to give widely used cell lines such as the 3T3-L1 pre-adipocyte line (Green and Meuth, 1974).

RCJ 3.1, a clonal population of osteoblast-like cells derived from a late stage sequential collagenase digest of foetal rat calvaria (chapter 1.9a), differentiated spontaneously in the presence of 15% foetal calf serum, dexamethasone, ascorbate and β -glycerophosphate to give four distinct mesenchymal

phenotypes, multinucleate muscle cells, adipocytes and discrete nodules exhibiting a chondrogenic or an osteoblastic morphology (Grigoriadis et al, 1988). 164 subclones were prepared and differentiation was observed in 31%, but was largely restricted to one or two phenotypes only (Fig 1.6).

Contents of Clone.	Colonies - % of total.
Myotubes.	5%
Adipocytes.	9%
Chondrocytes	8%
Myotubes & Adipocytes.	1%
Myotubes & Chondrocytes.	5%
Adipocytes & Chondrocytes.	2%
Myotubes, Adipocytes & Chondrocytes.	1%
Fibroblastic cells.	69%

Fig 1.6. Analysis of differentiation potential of subclones cloned from the pluripotential, embryo rat calvarial derived clone RCJ 3.1, showing that most subclones capable of differentiation have a restricted differentiation potential when compared to that of the parent clone (Grigoriadis et al, 1988).

This suggested an increasing degree of commitment amongst the subclones, and a hierarchical relationship between the differentiated cells, the subclone precursors and the original RCJ 3.1 precursor.

1.13: CELLS CAPABLE OF BEING INDUCED TO FORM BONE IN OTHER
 STROMAL SYSTEMS.

1.13a: DOPC'S AND IOPC'S.

Transitional bladder epithelium, explanted into the muscles of the rectus sheath, can induce ectopic ossification (Huggins, 1931; 1968; Huggins and Sammett, 1933). Marrow stromal cells, implanted in vivo in DC's form a bone-like matrix, whereas stromal cells from extra skeletal tissues such as the spleen or thymus did not. When implanted into DC's with transitional bladder epithelium, however, both thymus and spleen stromal tissue formed bone (Friedenstein 1968; 1973; 1976). On the basis of these observations two categories of osteogenic precursors were proposed, a determined osteogenic precursor cell (DOPC), capable of forming bone without an osteoinductive stimulus, and an inducible osteogenic precursor (IOPC), found in extra skeletal sites and capable of forming bone on induction by a specific osteoinductive agent (Friedenstein, 1973).

Two experimental models of osteoinduction in vivo have been described

- i) osteoinduction by epithelial cells
- ii) osteoinduction by demineralized bone matrix.

1.13b: OSTEOINDUCTION BY EPITHELIAL CELLS.

Huggins (1931) reported that bone formed wherever explanted transitional bladder epithelium came in contact with mesenchyme of the rectus muscle sheath. More recently, cultured epithelial cell lines have been found to be capable of osteoinduction (Wlodarski, 1985; Anderson 1976; 1990). The FL-amnion, transformed human epithelial cell line (Anderson et al, 1964), could induce bone on ectopic implantation into the thigh of immunosuppressed mice. Electron microscopic examination failed to demonstrate any significant matrix associated with the FL-amnion cells (Anderson, 1967), and supported the conclusion that contact between the epithelial cells and the host mesenchyme was an essential part of osteoinduction.

1.13c: OSTEOINDUCTION BY DEMINERALIZED BONE MATRIX.

Experimentally, it is well established that demineralized bone matrix can induce ossification when implanted ectopically in vivo (Urist 1965; 1989) in a process which histologically resembles endochondral ossification (Reddi, 1972). The osteoinductive activity appears to be mediated by a protein or proteins collectively termed bone morphogenic protein or BMP.

A single protein capable of osteoinduction has not yet been identified, however, recent work has localized BMP activity in rat bone to seven novel proteins termed BMP1 to 7 (Wozney et

al, 1988, 1989), related to the TGF- β superfamily. Preliminary studies have indicated that one protein, BMP 2, may have some activity on relatively undifferentiated cells.

1.13d: OSTEOINDUCTION - CONCLUSIONS.

Both the osteoinductive agent and its target cell remain unidentified. The favoured candidate for the target cells are the small 'adventitial' or 'reticular' cells that accompany small blood vessels (Ham and Cormack, 1979; Owen and Friedenstein, 1988).

It is unknown if the osteoinductive stimulus by explanted epithelial cells is the same as that from demineralized bone matrix. An attractive although speculative idea is that these examples are analogous to different processes in vivo. Epithelial induced osteogenesis may be analogous to the epithelial-mesenchymal interactions seen during development whilst matrix induced osteogenesis resembles a process of endochondral ossification seen during later bone growth and repair once growth has ceased. Investigation of these interactions are likely to be of great interest as they may direct future research towards an understanding of the factors promoting osteogenic differentiation in early stem cells.

1.14: OSTEOGENIC DIFFERENTIATION IN VITRO.

Although in vivo assay systems have provided valuable

qualitative data, they are unsuited to the investigation of specific factors promoting differentiation in an osteogenic direction. With in vitro systems there is the potential for controlling culture conditions. However, to date, the in vitro culture systems supporting osteogenic differentiation require the use of media which is ill defined, completed with up to 30% serum. Furthermore, care must be taken to ensure that any mineralized tissue formed reflects osteoblastic differentiation rather than dystrophic calcification. Nevertheless, useful in vitro models of osteogenesis have been developed. Osteoblast-like cells, isolated from foetal rat or murine calvaria and cultured to confluence in medium with 20% foetal calf serum, hydrocortisone or dexamethasone, β -glycerophosphate and ascorbate form a multilayered structure characterized by discrete mineralizing nodules (Ecarot-Charrier et al, 1983; Bellows et al, 1986) lined by a layer of osteoblast-like, AP positive cells. The collagen components of the mineralized matrix were mostly collagen type I and further characterized by the presence of the bone matrix proteins osteocalcin and osteonectin (Aubin et al, 1990; Bellows et al, 1986; Maniatopolous et al, 1988). At an electron microscope level, matrix vesicles were seen with evidence of membrane bound AP activity. The number of nodules formed bore a linear relationship to the number of cells plated, and it was concluded that the nodules were clonal, and each reflected the progeny of a single CFU-F. Statistical analysis showed that approximately 1:300 plated cells had the capacity to form a nodule (Bellows and Aubin, 1989; Aubin et al, 1990).

Confluent cultures of human osteoblast-like cells form mineralized nodules (Beresford et al, 1991). Mineralized nodule production required the replacement of ascorbate in the culture medium with L-ascorbate-2-phosphate, an ascorbate analogue with an extended half life in culture (Hata and Sudo, 1989). This technique, however, has only just been developed and has not yet been applied to the study of osteoblast ontogeny.

Adherent stromal cells from adolescent rat femoral midshaft marrow formed bone-like nodules in vitro, furthermore, when the matrix was isolated and implanted in vivo it had osteoinductive potential (Maniatopoulos et al, 1988).

1.15: AIMS OF THE PROJECT.

A major objective of this project was to obtain information about the hierarchy and potential for differentiation of the cell lines of the marrow stromal system in young adult New Zealand White rabbits. (Owen, 1978; 1985) (chapter 1.3). Specific aims were as follows;

- 1) To investigate the conditions necessary for the isolation and growth of clonal populations of adipocytic stromal cells in vitro (chapter 4),
- 2) To test their differentiation potential in vivo using a diffusion chamber assay (chapter 4),

- 3) To compare their differentiation potential with that of clonal populations of fibroblastic marrow cells in vivo (chapters 3 and 6).

Extra-medullary adipocytes have been studied extensively and stage specific phenotype differentiation markers defined at the protein and mRNA levels. Research into the marrow stromal system has been hampered by a lack of phenotype specific differentiation markers. A further objective was to test if a stage specific marker of extra-medullary adipocyte differentiation, the PAL422(AP2) protein, was expressed in marrow adipocytes. Because the PAL422(AP2) specific antiserum was raised in rabbits, a rat marrow stromal model had to be used (chapter 5).

CHAPTER 2. MATERIALS AND METHODS.

2.1: IN VITRO CULTURE OF MARROW CELLS AND ISOLATION OF SINGLE COLONIES.

2.1a: PREPARATION OF MARROW STROMAL CELL CULTURES.

Marrow cells were flushed from the midshafts of femora of male NZW rabbits, 800-1000g body weight, or inbred female PVG/Olac rats (Olac Ltd. Bicester, UK), 90-110g body weight, with serum free BGJb medium (GIBCO/BRL). A suspension of single cells was made by mechanical disaggregation by passing through 19, 21, and 23 gauge needles in sequence. Any remaining cell aggregates were removed by passage of the cells through 90 μ m nylon mesh (Bolting cloth, H. Simon Ltd, Stockport, UK). Samples of the cell suspension were diluted with 0.5% (w/v) Trypan Blue dissolved in 0.16 M-ammonium chloride and the number and viability of nucleated cells counted in a haemocytometer. 5×10^4 to 10^7 cells were plated out in 25 cm² plastic tissue culture flasks (Nunc, GIBCO/BRL.) and cultured in BGJ_b medium with ascorbate (0.05 mg ml^{-1}), and glutamine (0.1 mg ml^{-1}), (referred to subsequently as BGJ_b) at 37°C in a 5% CO₂ in air humidified incubator. For the establishment of discrete stromal colonies or adherent cultures BGJ_b was supplemented with either 20% (v/v) foetal calf serum (FCS) (GIBCO/BRL), or 15% (v/v) autologous rabbit plasma (RbP) or 15% rat plasma (RtP) obtained from syngeneic animals. In some experiments hydrocortisone (hemisuccinate salt, Sigma Chemical

Co Ltd) was added to give a concentration of 10^{-7}M (Hc), or dexamethasone to give a concentration of 10^{-8}M (DEX), to selected flasks. In other cultures marrow cells were plated out initially in BGJb and incubated undisturbed for 2 hours. Non-adherent cells were then removed, the adherent cells washed twice with PBS and medium completed with serum added. This procedure is subsequently referred to as pre-incubation.

For subculture and passaging, cells were harvested after incubation for 8 minutes at room temperature with 0.05%(w/v) trypsin-EDTA.

2.1b: PREPARATION OF RABBIT PLASMA (RbP) AND RAT PLASMA (RtP).

Autologous RbP was prepared from 20 to 30 ml of blood obtained by direct cardiac cannulation into a syringe pre-treated with 50 iu of heparin. Up to 10ml of blood was obtained from individual rats by direct cardiac cannulation into a syringe pre-treated by flushing through with heparin leaving the void volume remaining. Samples were spun at 2500g for 20 minutes at 4°C and the supernatant collected (Slichter, 1985). Samples of plasma were freshly prepared immediately before use.

To prepare rat serum, blood was collected by direct cardiac cannulation and left to clot overnight at 4°C. The clear supernatant was collected and stored at -20°C.

2.1c: AUTOLOGOUS IRRADIATED FEEDER CELLS (IRF).

In some experiments cells were cultured in the presence of 2×10^7 prepared from autologous marrow cells given 3000 Gy by τ -irradiation from a Caesium source.

2.1d: FIXATION OF CULTURES.

After 14 to 17 days cultures selected for fixation and observation, alkaline phosphatase (AP) localization or immunocytochemistry were washed twice in Phosphate Buffered Saline (PBS) at 4°C, fixed in 95% ethanol at 4°C for 1 minute, washed again in 3x distilled water and allowed to dry in air. Cultures to be stained with Oil-Red-O for lipid were fixed in formol calcium.

2.1e: COLONY COUNTING AND SCORING FOR ADIPOGENESIS AND ALKALINE PHOSPHATASE (AP) ACTIVITY.

Cultures were stained to localize AP activity using a commercial kit (Sigma Kit no 85, Sigma Chemical Co Ltd). Fibroblastic colonies, cultured in medium supplemented with FCS were counted using an Anderman counter as previously described (Owen et al, 1987). Colonies >1.0mm diameter were measured and designated by eye into one of five categories, ranging from 0 to ++++ for AP activity according to the amount and intensity of the staining reaction.

In cultures where the number of adipocytes and fibroblasts were being compared, colonies >0.2mm in diameter were counted and measured in vitro under phase contrast using a Zeiss inverted microscope x100.

Adipogenesis was assessed in vitro according to the proportion of the area of a colony which contains cells with refractile lipid inclusions as seen under phase contrast microscopy, and the results expressed per colony using a scale 0 to +++++. Details of the scale with photomicrographs of examples are given in chapter 4.2 c and d.

2.2: AUTORADIOGRAPHIC STUDIES ON TRITIATED THYMIDINE UPTAKE BY COLONIES IN VITRO.

Flasks with discrete single colonies were prepared as described in section 2.1. On day 12 of culture ^3H Thymidine (Amersham International, Amersham, Bucks UK) was added to each flask at a dose of $2.25\mu\text{Ci}$ per ml of culture medium, incubated at 37°C with 5% CO_2 in air for 2 hours, washed in cold PBS then twice in 1mg ml^{-1} non-radioactive thymidine in 70% alcohol at 4°C before 1 minutes fixation in 95% ethanol at 4°C . Flasks were then rinsed in 3x distilled water and allowed to air dry then stained to localize AP activity. The base of the flask, ie; the tissue culture surface, was cut away from the body of the flask and prepared for autoradiography using Kodak AR10 stripping film. Pieces of film $1\frac{1}{2}\text{cm}^2$ were stripped and floated face down on clean distilled water at about 23°C . After 10

minutes, when the film had expanded sufficiently, it was picked up and used to cover the surface of the flask so that the emulsion side was in contact with the radioactive colonies. After drying, the flask base and film were left to expose at 4°C in a sealed black box. After 2 to 3 weeks exposure the film was developed and fixed at 18°C then examined x25 or x100 using a Zeiss Axiophot microscope.

2.3 PREPARATION OF CELL POPULATIONS FROM SINGLE COLONIES.

2.3a: COLONIES ISOLATED BY RING CLONING.

Well separated single colonies were obtained for ring cloning by inoculating 5×10^4 to 10^6 rabbit whole marrow cells per 25cm² flask.

Fibroblastic colonies were established in BGJb supplemented with FCS, Hc and IRF. The cultures were re-fed at 10 days and again on every 3rd day thereafter with the same medium and serum supplement but excluding the IRF.

To establish adipocytic colonies, marrow cells were plated initially in BGJb medium and incubated for 2 hours as described in section 2.1a, before the addition of medium supplemented with autologous RbP and Hc. The cultures were scanned by phase contrast microscopy using a Zeiss inverted microscope x100 on day 10. The position of discrete adipocytic

colonies suitable for ring cloning was noted using the vernier scale attached to the microscope stage so that they could readily be located again. The culture medium was then changed to BGJb supplemented with FCS and Hc, with further changes at three or four day intervals.

In the case of both fibroblastic and adipocytic cultures, individual colonies greater than 6mm in diameter were subcultured after 19 to 24 days using a cloning ring. Cloning rings were milled from pieces of perspex rod¹ 1.6mm diameter, hollowed out to give a ring of internal diameter 1.2mm and 2mm deep. The rings were reusable and sterilized with ethylene oxide. Trypsinized cells were counted in a haemocytometer and transferred to a 2cm² well in a 24 well tissue culture plate. Growth medium in secondary culture was α MEM (Flow Laboratories Ltd) supplemented with 20% FCS. The cells were then expanded through several passages in the wells, and into 25cm² flasks (1:2 split ratio) until sufficient cells for implantation in sealed diffusion chambers were obtained ($>2.0 \times 10^5$ cells).

2.3b: CLONAL POPULATIONS ISOLATED BY LIMITING DILUTIONS.

2×10^6 cells obtained from primary rabbit marrow cultures were plated out in 25 cm² flasks in BGJ_b with FCS and IRF. After 9

¹The assistance of the Department of Prosthetic Dentistry, The Eastman Dental Hospital and Institute of Dental Surgery, in the manufacture of the cloning rings is gratefully acknowledged.

days the subconfluent population of cells was washed twice in PBS, trypsinized, spun for 10 mins at 150 g, resuspended in 1 ml of α MEM and counted in a haemocytometer. Cells were plated in 96 well microwell plates. Cell numbers were adjusted to give 700 cells per well in the first of twelve rows, from which serial dilutions, 1:2, were used down to an average 0.3 cells per well in row 12. Growth medium was α MEM with FCS. Wells were screened using an Olympus CK 2 inverted microscope x60 after 48 hours, 6 days and 16 days just prior to the first medium change. Only single colonies growing in wells where the average inoculum was 1.5 cells per well or less were selected for expansion. After about 4 weeks selected larger colonies were trypsinized and sub cultured through several passages until $>2 \times 10^6$ cells had been obtained to give sufficient for two aliquots, one for implantation in sealed diffusion chambers and the other to give >3 samples for cryostorage.

2.4: CULTURE OF CELL LINES.

2.4a: ROS 17/2.8 AND UMR 106 CELLS.

Cells were reconstituted from cryostorage and grown to confluence in α MEM supplemented with 10% FCS. Cells were then trypsinized and 5×10^4 cells plated into 25cm^2 tissue culture flasks in α MEM completed with 20% FCS and 10^{-7}M Hc.

2.4b: 3T3-L1 CELLS.

Cells from this murine pre-adipocyte cell line were obtained from the European Tissue Culture Collection, Porton Down, Wiltshire. 10^5 cells were plated into 25cm^2 tissue culture flasks and cultured to confluence in α MEM supplemented 10% FCS. At confluence a regime designed to promote adipogenic differentiation was instituted (Bernlohr et al, 1984). Medium was changed to α MEM with 10% FCS, $100\mu\text{gml}^{-1}$ methyl isobutyl xanthine (MIX) and $7.2 \times 10^{-6}\text{M}$ porcine insulin. After 48 hours the medium was changed to α MEM and 10% FCS. Within 5 days adipogenic differentiation was seen microscopically.

2.5 IN VIVO ASSAY OF OSTEOGENESIS IN SEALED DIFFUSION CHAMBERS (DC'S).

2.5a: IN VIVO ASSAY OF OSTEOGENESIS.

Diffusion chambers (DC) assembled from commercially available components (Millipore Corporation) were 13mm external diameter, 9mm internal diameter, 2mm thick with approximately 127mm^3 capacity. The pore size of the membrane filters was $0.45\mu\text{m}$. Cells were inoculated into the chambers through a hole in the external ring which was then sealed with a tapered plastic plug coated with glue. A single DC was implanted intra peritoneally into a 20mg MF1/NuNu mouse under Saggatal (0.06mg/g body wt) and Ethrane anaesthesia. Chambers were harvested after 60 days fixed in 95% methanol and embedded in

Glycol Methacrylate as described by Ashton et al (1980).

2.5b: HISTOCHEMICAL ANALYSIS OF DIFFUSION CHAMBERS (DC'S).

Sections were routinely stained with Toluidine Blue, for alkaline phosphatase activity (Sigma kit No. 85) and the Von Kossa reaction.

2.5c: IMMUNOHISTOCHEMICAL ANALYSIS OF DIFFUSION CHAMBERS (DC'S).

Antisera.

Goat anti rabbit polyclonal antisera to collagen types I, II, III and V raised by Page et al (1986) were obtained². BRL-12 is a murine anti rabbit monoclonal antibody to the core protein of the bone and dentine specific rabbit keratan sulphate proteoglycan (Joyner et al, 1989, 1991).

Collagen Immunostaining.

Sections, fixed in 95% ethanol were demineralized in 0.3M EDTA, Ph 7.3, followed by pre-incubation in turn for 1 hour at 37°C with 1% trypsin, 2% hyaluronidase, 2% l-lysine in PBS and 4% bovine serum albumen in PBS with 10% heat inactivated rabbit serum. Between each inoculation sections were washed x3

²Gift of Dr Doreen Ashhurst, Department of Anatomy, St Georges Hospital Medical School, Cranmer Terrace, London SW17.

in PBS. Sections were incubated in antiserum at 4°C for 18 hours. Rabbit anti goat AP conjugated secondary antibody (Sigma Chemical Co. Ltd) was used to visualize primary antibody binding sites using standard histochemical techniques. Native antibody was blocked with levamisole (1mgml⁻¹). As controls, sections were stained with primary but no secondary antibody, with secondary but no primary antibody, or with normal goat serum replacing the primary antiserum.

BRL-12 Immunostaining.

Localization of keratan sulphate proteoglycan was carried out on sections fixed in 95% alcohol and embedded in glycol methacrylate as described by Joyner et al (1989)³.

2.6: IDENTIFICATION OF AN ADIPOCYTE SPECIFIC PROTEIN IN STROMAL CULTURES BY PROTEIN BLOTTING AND IMMUNOCYTOCHEMISTRY.

2.6a: PREPARATION OF CULTURED CELL EXTRACTS.

Primary cultures of adherent marrow stromal cells from rat and rabbit, cultures of the osteoblast like osteosarcoma cell lines ROS 17/2.8 and UMR 106, and cultures of the pre-adipocytic cell line 3T3-L1 which had been allowed to differentiate, were grown to confluence, washed twice in cold

³The assistance of Dr Clive Joyner with the BRL-12 staining is gratefully acknowledged.

PBS before being scraped into a small volume of HEPES buffered saline, pH 7.4, with 7.5 mgml^{-1} digitonin (Sigma Chemical Co. Ltd), hereafter called releasate. Following pulsed sonication for 30 seconds at 4°C , cell extracts were centrifuged at $1000g$ for 20 minutes. The supernatant was then precipitated in 10 volumes of 1:1 ethanol:diethyl ether at -20°C for 18 hours. Following centrifugation at $1500g$ for 30 minutes the supernatant was removed and the precipitate allowed to dry under a constant stream of nitrogen at room temperature. The precipitate was solubilized in 0.1% SDS and the protein content assayed using a commercially available kit (Bio-Rad Laboratories Ltd) based on the method of Bradford⁴. Protein extracts were stored at -20°C .

2.6b: PREPARATION OF MARROW EXTRACTS AND EXTRA MEDULLARY
FAT EXTRACTS.

Peri-abdominal fat samples obtained from freshly killed NZW rabbits or PVG/Olac rats were minced and collected at 4°C in 2mls of releasate. Tissue was homogenized for 1 minute in releasate in 10 second pulses, sonicated, then centrifuged at $1500g$ for 30 minutes. The liquid cell suspension was collected discarding the supernatant fat.

Marrow was prepared by flushing midshaft marrow out of freshly obtained femurs from 100g rats, adolescent (1kg) or adult (3kg) rabbits with 2mls of releasate. Following

⁴Bradford M., Anal. Biochem., V72., Pg248., (1976).

homogenization, sonication and centrifugation as above, the cell suspension was collected. Tissue extracts were processed as described for the cultured cell extracts (section 2.6a).

2.6c: ANTISERUM.

A rabbit polyclonal antiserum⁵ to a synthetic peptide was used, corresponding to amino acids 1 to 12 from the amino terminus of 3T3-L1 derived pAL422(AP2) protein, an intracellular fatty acid binding protein widely accepted as a specific protein marker of differentiation in an adipocytic direction (Bernlohr et al, 1984; 1985; Lane et al, 1990; Ailhaud et al, 1987).

2.6d: GEL ELECTROPHORESIS AND PROTEIN BLOTTING.

Samples of cell and tissue extracts containing similar quantities of protein, in the range 30µg to 50µg, were run on 8-18% (w/v) polyacrylamide gradient gels, together with pre-stained protein molecular weight markers (Bio-Rad Laboratories Ltd). Some tracks were stained with coomassie blue stain, then destained in 40% methyl alcohol with 10% glacial acetic acid to remove the background. Others were blotted onto a 0.2µm pore size nitrocellulose membrane using a Bio-Rad Trans-blot cell (Bio-Rad Laboratories Ltd) and a blotting buffer consisting of 192mM glycine, 25mM tris and 10% (w/v) methanol

⁵Kindly donated by Dr M. D. Lane, Department of Biological Chemistry, The Johns Hopkins School of Medicine, Baltimore, Md USA.

made up to pH 8.3.

Blots were blocked overnight in PBS containing 0.05% tween (PBS/Tween) at 4°C, then incubated for 1 hour in 10% FCS in PBS/Tween at 4°C to block non-specific binding. Blots were then incubated for 1 hour in a 1:1000 dilution of antiserum at room temperature. Following repeated washing (at least 6 x 5 minutes) in PBS/Tween, blots were incubated for 30 minutes at room temperature in swine anti-rabbit secondary antibody, 1:900 dilution. They were then washed again in 1:400 rabbit PAP (peroxidase-anti-peroxidase conjugated antibody). Colour was developed using the DAB (tetra-aminobiphenyl hydrochloride) reaction. 100µl of 30% hydrogen peroxide was added to 50 mg of DAB in 50 ml of 0.1M imidazole buffer (pH 7.3), freshly made up in the dark immediately before use. The blot was soaked in the hydrogen peroxide/DAB solution for about 15 seconds until the colour developed. The reaction was then stopped by repeated washing in distilled water, and the blot allowed to dry at room temperature in air.

Details of positive and negative controls are given with the results in chapter 5.2 b,c and d.

2.6e: IMMUNOHISTOCHEMISTRY.

To localize AP2 protein in situ, flasks were fixed in ethanol as described in section 2.1d. The immunostaining protocol was

essentially the same as that used to localize AP2 following Western Blotting except that the primary antiserum was used at a dilution of 1:500 rather than 1:1000. Details of experimental controls are included with the results.

2.7: mRNA EXTRACTION FROM RAT MARROW STROMAL CULTURES AND NORTHERN BLOTTING.

2.7a: CELL CULTURE PREPARATION.

Single marrow cell suspensions were prepared as described (section 2.1). 4×10^7 cells were plated into 25cm² tissue culture flasks in α MEM (GIBCO/BRL) supplemented with 15% FCS and 0.5mgml⁻¹ ascorbate kept at 37°C in a humidified 5% CO₂ in air incubator. DEX was added to some flasks. The medium was changed after 5 days. At confluence, after 8 to 10 days, cells were washed x2 with PBS and treated sequentially with collagenase (Type VII - Sigma Chemical Co. Ltd - product no G0773) in serum free medium for 60 minutes at 37°C, and 0.5% trypsin with 0.1% EDTA (GIBCO/BRL) in PBS, pH 7.2, for 5 minutes at 37°C. Following gentle centrifugation at 200g and 4°C for 10 minutes, cells were counted in a haemocytometer before being plated in 80cm² tissue culture flasks at a density of $4.5 \times 10^3 \text{cm}^{-2}$. Cells were left to settle for 24 hours before the addition of ascorbate or ascorbate with DEX. Three flasks were prepared for each culture condition. Cultures were incubated for a total of 8 days with changes of medium every 2 or 3 days.

2.7b: RNA EXTRACTION.

The preparation of RNA follows the method described by Leboy et al, (1989). Cells were washed twice in cold PBS, released with a rubber policeman and extracted in a guanidinium isothiocyanate buffer (Ph 7.0) containing 25mM sodium citrate, 0.5% sarkosyl (sodium lauryl sarcosinate), 0.1M β -mercaptoethanol and 4M guanidinium isothiocyanate to give a total of 4ml of extract per group of flasks for each experimental condition. Total cellular RNA was prepared from the homogenate by extraction in sodium acetate saturated phenol at 55°C, re-extracted with chloroform, and the nucleic acid precipitated with 1.0 volumes of isopropyl alcohol then centrifugation at 12,000g for 15 minutes. The pellet was resuspended in 7.5M guanidine HCl. Following the addition of 3M sodium acetate pH 5.2 to give a final concentration of 0.3M, nucleic acids were re-precipitated with 2.5 volumes absolute ethanol at -20°C for 2 to 4 hours. This pellet was resuspended in 50-100 μ l of DEPC-H₂O (diethyl pyrocarbonate - 0.1% in H₂O) pH 7.5 and the amount of RNA produced quantitated by measuring the UV absorption of a diluted sample at 260 μ m. Yields were typically 200-600 μ g of total RNA per group of 3 flasks. Samples were divided into aliquots and stored at -70°C as a precipitate in ethanol.

2.7c: HYBRIDIZATION PROBES;

The pAL422 (AP2) probe was a 0.7Kb fragment of DNA derived

from an 3T3-L1 adipocyte cDNA library, encoding a 132 amino acid protein of 14.6KDa, and inserted into a pGEM blue vector (Bernlohr et al 1984)⁶. The rat collagen type I⁷ was a 1.6Kb fragment inserted into the Pst-1 site of a pBR322 vector and spanning approximately 500 amino acid residues within the triple helical region of the $\alpha 1(1)$ chain (Genovese et al, 1984). Both probes were labelled with ³²P by nick translation using a commercial kit (GIBCO/BRL) and α [³²P]-dCTP to specific activities of at least 5×10^7 cpm/ μ g DNA.

2.7d: NORTHERN BLOTTING.

20 μ g RNA from each sample was suspended for 1 hour in 10 μ l of glyoxal denaturing buffer at 50°C. Samples were loaded on to 1% agarose gels with RNA standards (GIBCO/BRL) for electrophoresis using a 0.01M NaPO₄ electrophoresis buffer, pH 6.5. Following electrophoresis the agarose gel was divided and the portion with the RNA standards stained with ethidium bromide (0.5 μ gml⁻¹ in 50mM NaOH), destained in 0.1M ammonium acetate and photographed using ultraviolet light. The remaining portion was blotted onto 'Zetaprobe' nylon membranes (Bio-Rad Laboratories Ltd) by capillary elution under alkaline conditions, using a 7.5mM NaOH blot buffer as described by Leboy et al, (1988) followed by a 10 minute wash in x2 SSC (sodium citrate pH 7.0) with 0.1% SDS. Blots were dried

⁶Gift of Dr M. D. Lane, Department of Biological Chemistry, The Johns Hopkins School of Medicine, Baltimore, MD USA.

⁷Gift of Dr B. Kream, University of Connecticut, Hertford, Connecticut, USA.

briefly using absorbent toweling before storage wrapped in clingfilm at -20°C.

2.7e: HYBRIDIZATION.

Pre-hybridization was carried out at 42°C in 4xSSPE (standard saline phosphate EDTA, Ph 7.4) with 50% formamide, 300µgml⁻¹ salmon sperm DNA, 0.5% dried non-fat milk (Blotto) and 1% SDS. Hybridization was carried out overnight with identical buffer with the addition of 5-10x10⁶ cpm of probe as described by Leboy et al, (1988). Several post hybridization washes were carried out in x0.1 SSC with 0.1% SDS at 42°C. An additional wash at 50°C was performed when probing for collagen type I to prevent cross hybridization to ribosomal RNA's (Leboy et al,, 1991). Hybridization was visualized by exposing the blot to Kodak X-OMAT AR film at -70°C for up to 7 days. Blots were stripped for re-blotting by rapid boiling in 1% SDS, washed, dried and stored as described at -20°C.

CHAPTER 3. FIBROBLASTIC COLONIES CULTURED FROM MARROW
CELLS.

3.1: INTRODUCTION.

In order to study lineage relationships it is essential to have a suitable assay system to test the differentiation potential of individual clonal populations. Amongst the most widely used have been those in which cells were transplanted to ectopic sites and the tissue formed observed histologically. By implanting cells in vivo in sealed diffusion chambers (DC) they could be kept separate from those of the host, yet cell growth was able to take place in the physiologic milieu of the host animal (Metchinkoff, 1887; Algire et al, 1954; Duthie, 1959).

The osteogenic potential of fresh rabbit marrow cells and of cultured adherent marrow fibroblasts has been well documented. These cells form osteogenic tissue when implanted in DC's or in an open system in vivo (Friedenstein, 1976; Friedenstein et al, 1982; Ashton et al, 1980; Bab et al, 1986). Tissue formed in DC's is not strictly bone as it lacks a blood supply and is not innervated. Additionally the mechanical environment of bone cannot be reproduced in a DC. Nevertheless, the mineralizing matrix formed within a DC resembles bone and cartilage at a light microscopic or an ultrastructural level, and stains immunocytochemically for collagens and bone specific proteins (Ashhurst et al, 1990; Joyner et al, 1989;

1991; Mardon et al, 1987; Bruder et al, 1990c). Osteogenic differentiation can occur within a DC making the technique a valid assay of osteogenic potential when studying the osteoblast lineage.

When marrow cell suspensions are cultured in vitro with foetal calf serum, fibroblastic colonies are formed (Owen et al, 1987; Friedenstein et al, 1987). Studies with tritiated thymidine labelling, karyotypic analysis of colonies in cultures of mixed male and female cells and time lapse cinematography provided strong circumstantial evidence that each colony is derived from a single cell (Friedenstein, 1976). It has been shown that, when tested in vivo in DC's, a proportion of the clonal cell populations, each derived from a single fibroblastic colony formed a mineralizing matrix resembling skeletal tissue (Friedenstein et al, 1987).

The purpose of this chapter is to

- 1) define conditions for the growth of discrete single fibroblastic colonies in vitro and to assay their osteogenic potential in vivo in DC's,
- 2) characterise the extra cellular matrix of the tissue formed in DC's from single colonies and confirm that single colonies can form a mixture of tissues,
- 3) provide data on fibroblastic colonies for comparison with similar studies made on adipocytic colonies (chapter 4).

3.2: RESULTS.

3.2a: STROMAL COLONIES FORMED IN ADHERENT MARROW STROMAL CULTURES.

Marrow cells cultured in BGJb supplemented with 10% or 20% FCS gave rise to colonies with a typical fibroblastic appearance. In preliminary experiments it was clear that colony forming efficiency (CFE) or the number of colonies formed per number of cells inoculated varied between animals and between batches of serum. Experiments were, therefore, designed to allow comparison between groups where cells from a single donor, cultured with serum from the same batch, were used. Under these conditions similar trends were observed in different experiments although the absolute values varied.

3.2b: CFE AND CELL INOCULUM.

The relationship between the number of colonies formed and the initial plating density is shown in Fig 3.1. The calculated values of CFE over the range 10^6 to 1.2×10^7 cells per flask are also shown. The CFE decreased with decreasing inoculum. If the number of colonies formed had been directly proportional to the inoculum the CFE would have been approximately constant. The fact that this was not the case implies that CFE does not depend simply on the number of colony forming cells inoculated (see discussion).

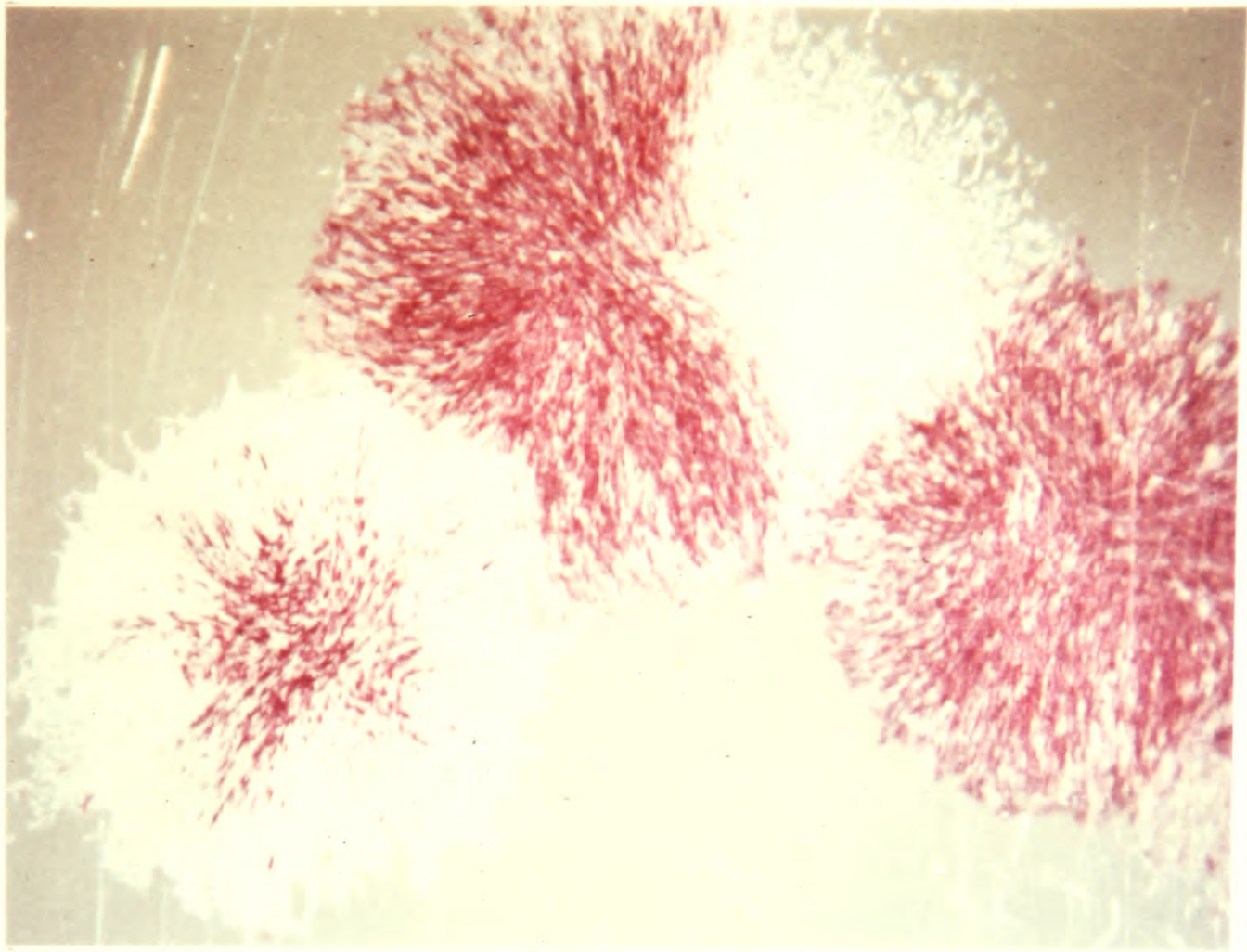


Plate 3.1.

Colonies cultured in BGJb, FCS and 10^{-7} M Hc, which are heterogeneous with respect to AP activity ($\times 10$). These examples are the work of Dr M. E. Owen.

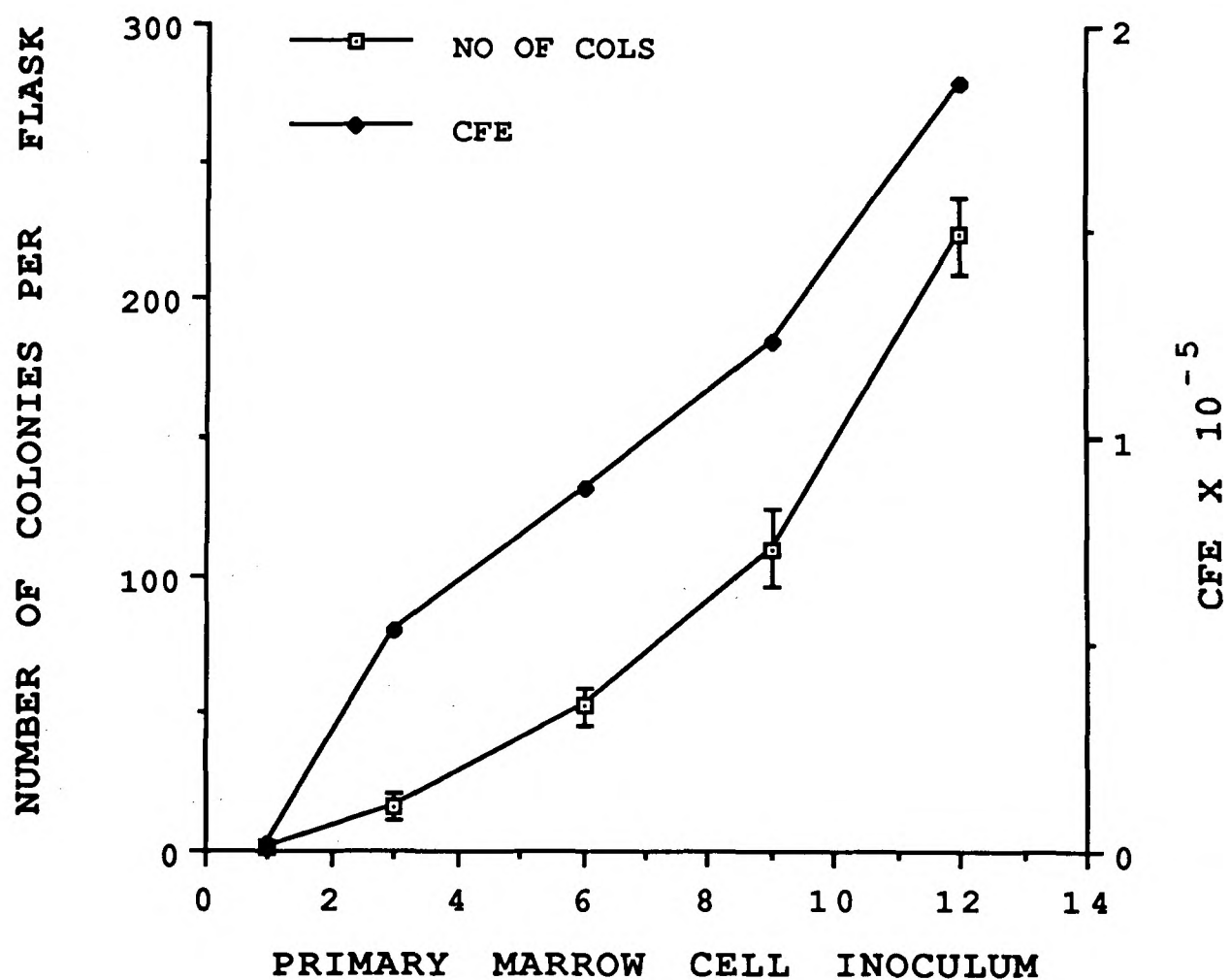


Fig 3.1

Colonies formed per 25cm^2 flask (Mean $\pm$ SD) plotted against the number of whole marrow cells inoculated. Calculated values for CFE are shown.

3.2c: CFE AND % SERUM SUPPLEMENT.

The CFE was increased when the percentage serum supplement was raised from 2% to 20% or 30% FCS (Fig 3.2). At the higher marrow cell inoculum required to achieve colony growth at 2% serum, cultures rapidly became confluent at 20% or 30% serum, thus in Fig 3.2, comparison between different serum supplements was made over a range of inocula.

Experiment	Inoc'n	CFE		
		2% FCS	20% FCS	30% FCS
5.6.87	7.2	0.43(0.03)	ND	ND
	4.3	0.16(0.04)	3.0(0.32)	ND
	2.0	0.0	2.8(0.36)	ND
	1.0	ND	0.65(0.02)	ND
19.6.87	18.0	0.43(0.04)	ND	ND
	12.0	0.46(0.07)	ND	ND
	8.0	0.19(0.08)	ND	ND
	5.0	0.0	ND	0.8(0.12)
	2.0	ND	ND	0.2(0.12)
	1.0	ND	ND	0.08(0.1)
17.7.87	2.5	0.32(0.06)	1.9(0.12)	ND
	1.3	0.38(0.08)	2.5(0.44)	ND
	1.0	0.23(0.15)	2.3(0.12)	ND

Fig 3.2 Results from three experiments comparing CFE x10⁻⁵ for equivalent inocula of marrow cells from the same donor cultured in BGJb medium to day 17 with different FCS supplements. Medium changes were on days 7, 11 and 14. Inoc'n is the primary nucleated marrow cell inoculum x10⁶. ND = not done.

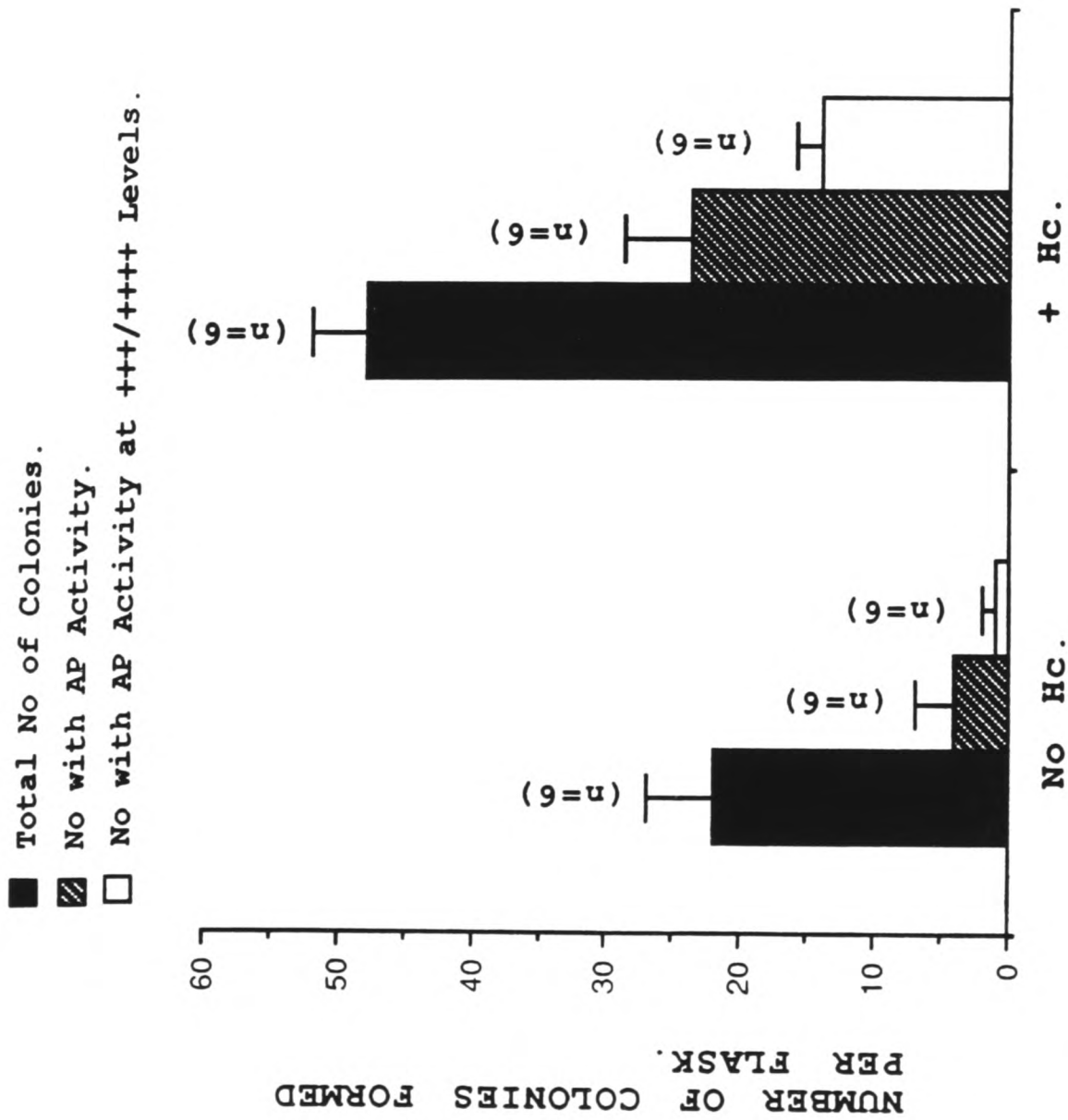


Fig 3.4

Colonies cultured in 10% FCS $\pm 10^{-7}$ M Hc were fixed on day 17 and AP activity localized. The number of colonies, the number with AP activity and the number showing AP activity at the +++/++++ levels are compared. (n = no of flasks)

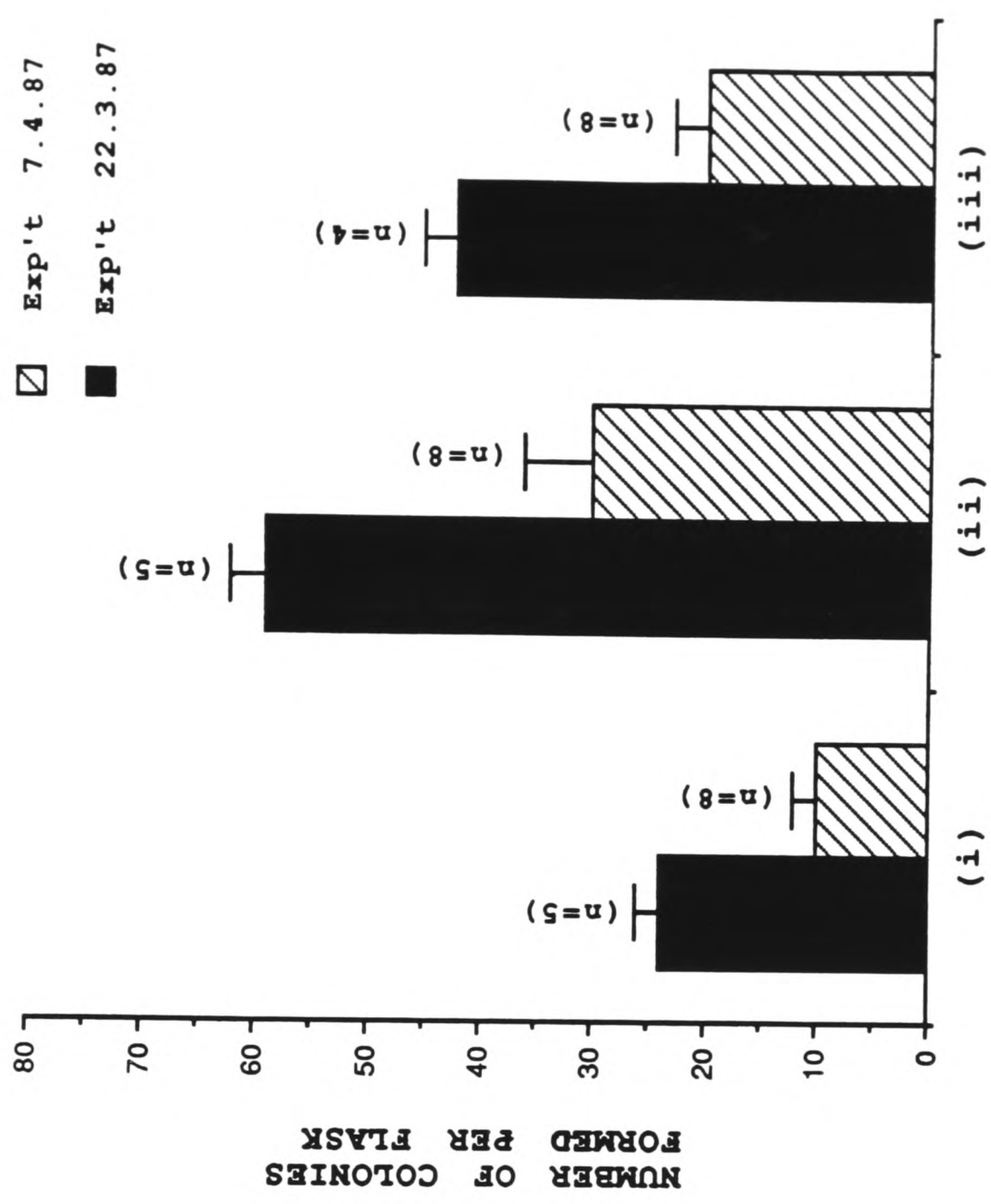


Fig 3.3

No of colonies formed per flask (Mean $\pm$ SD) after culture for 17 days in BGJb & FCS, comparing medium changing regimes. In Gp(i), medium was changed on days 7, 11 and 14; Gp(ii), on day 14 and in Gp(iii), the medium was left unchanged. (inocula per 25cm² flask; Exp't 22.3.87-7x10⁶ cells; Exp't 7.4.87-8.5x10⁶ cells) (n = no of flasks)

3.2d: CFE AND MEDIUM CHANGING REGIME.

The effect of the number of medium changes on CFE is shown in Fig 3.3 for two representative experiments. In Fig 3.3 the results obtained for three complete medium changes on days 7, 11 and 14 (Fig 3.3 (i)), were compared to the results for one change on day 14 (Fig 3.3 (ii)) or no medium change at all (Fig 3.3 (iii)). Flasks were fixed on day 17. The number of colonies obtained with one change on day 14 (Fig 3.3 (ii)) was greater than with no change at all (Fig 3.3 (iii)) and considerably greater than with changes on days 7, 11 and 14 (Fig 3.3 (i)). In the following experiments the medium was left unchanged to day 14 and changed every 5 days thereafter.

3.2e: CFE AND HYDROCORTISONE (HC).

The presence of 10^{-7} M Hc increased the number of stromal colonies formed, and the intensity of AP expression within each colony. Plate 3.1 shows a group of colonies following fixation on day 14 which vary with respect to size and histochemical expression of AP activity. The results of a single representative experiment in which colonies were grown in medium and 10% serum for 17 days with or without Hc is given in Fig 3.4. The addition of Hc increased the number of colonies per flask from 22 ± 5 to 48 ± 4 . 50% of those cultured with Hc were AP +ve compared to 18% in the absence of Hc. Furthermore, the proportion of colonies expressing AP activity at the highest staining levels (+++ or +++) increased from 5%

to 29% in the presence of Hc. These results are in good agreement with previous findings (Owen et al, 1987).

With or without Hc, colonies were composed of fibroblastic cells. Refractile cytoplasmic lipid vacuoles were very rarely seen in rabbit marrow stromal colonies cultured under the conditions described with the FCS batches used.

3.2f: CFE AND IRRADIATED FEEDER CELLS (IRF).

An absolute requirement for the isolation of discrete single colonies for ring cloning is that they should be well spaced i.e. at a density of 2 or 3 per 25cm² culture flask. From Fig 3.1 it was apparent that the number of colonies decreased with primary marrow cell inoculum in a non-linear fashion and at low marrow cell inocula it was not possible to obtain colonies with a frequency of one or two per flask consistently.

In other systems the presence of irradiated feeder cells (IRF) has made it possible for colonies to grow at low seeding density. The effect of the addition of 2×10^7 autologous IRF on CFE for a range of cell inocula was investigated both with and without an initial 2 hour pre-incubation of the marrow cell inoculum in serum free medium. Colonies were counted after 17 days in culture. With no pre-incubation the CFE in the presence of IRF was increased (Fig 3.5 columns (i) and (ii)). In particular, the CFE was increased at low inocula and was

approximately constant over the range tested. In the presence of IRF, therefore, it was possible to get a small number of well spaced, discrete, single colonies suitable for ring cloning.

Marrow cells were pre-incubated for 2 hours in serum free medium, the non-adherent cells were removed and medium with 20% FCS and Hc with or without IRF was added. In the absence of feeders CFE was reduced by >80% (Fig 3.5 column (i) and Fig 3.5 column (iii)), whilst in the presence of IRF, CFE was partially restored (Fig 3.5 column (ii) and Fig 3.5 column (iv)).

On the basis of these observations, medium with a 20% FCS supplement, 10^{-7} M Hc and 2×10^7 autologous IRF were determined to be the best conditions for culturing single colonies suitable for ring cloning.

Exp't.	Inoc'n	No 2hr Pre-incubation.		With 2hr Pre-incubation.	
		Column (i) BGJb+20%FCS +Hc.	Column (ii) BGJb+20%FCS +Hc+IRF.	Column (iii) BGJb+20%FCS +Hc.	Column (iv) BGJb+20%FCS +Hc+IRF.
30.3.88	4.0	1.7(0.25)	3.5(0.02)	0.31(0.02)	2.4(0.13)
	1.0	0.02(0.05)	3.1(0.71)	0	1.0(0.13)
	0.6	0	2.9(0.91)	ND	ND
17.11.87	2.8	0.79(0.05)	1.55(0.15)	0.11(0.03)	0.78(0.09)
	0.7	0.24(0.15)	2.19(0.36)	0	0.19(0.22)
22.4.88	3.0	1.9(0.23)	*	ND	ND
	1.0	1.9(0.58)	8.3(1.6)	ND	ND
	0.5	0.2(0.2)	8.4(1.7)	ND	ND
	0.2	0	4.3(1.1)	ND	ND

Fig 3.5. Comparison of the Number of Colonies Formed (Mean(SD)) in BGJb medium, 20% FCS, Hc, with or without IRF for three comparable experiments. Inoc'n is the number of whole marrow cells inoculated per 25cm² flask x10⁶.
* = flasks too confluent to count.

3.2g: ISOLATION OF COLONIES AND IMPLANTATION IN DIFFUSION CHAMBERS (DC'S).

By using a range of low cell inocula, from 5×10^4 to 10^6 cells per 25cm^2 flask in the presence of IRF, sufficient numbers of well spaced colonies suitable for ring cloning were obtained from several experiments. A summary of data for colonies isolated and subsequently expanded for the diffusion chamber assay is given in Fig 3.6. Of 28 isolated, 8 gave enough cells for implantation whilst the remainder failed to provide sufficient cells and were discarded. There were no significant differences either in colony diameter, the number of cells present or in the number of days in culture at the time of isolation between implanted and discarded colonies (Fig 3.6). However, on average, discarded colonies survived fewer passages.

	Implanted.	Discarded.
No of Colonies.	8	20
Diam (mm) at time of isolation.	6.9(2.2)	6.8(1.8)
No. of Cells at time of isolation.	0.54(0.33)	0.81(0.62)
No. of days to isolation.	19.8(4.2)	22.9(6.1)
No. of passages before being implanted /discarded.	4-6	1-4
No. of Cells inoculated per DC.	7.8(4.2)	----

Fig 3.6 Data for fibroblastic colonies isolated by cloning rings and expanded for implantation in vivo in DC's. Results are given as a Mean(SD) or as a range. The number of cells at the time of isolation and the number implanted into DC's are given x 10⁵.

3.2h: MORPHOLOGY OF TISSUE FORMED IN DIFFUSION CHAMBERS.

Histology.

Histological and immunocytochemical observation on the eight implanted chambers are summarized in Fig 3.7. Three chambers contained a mixture of bone, cartilage and fibrous tissues. The other five chambers contained fibrous tissue only. Plate 3.2 a,b,c and d shows photomicrographs of serial sections from one colony, Colony 46, which formed a mineralizing matrix

in vivo . Alkaline phosphatase activity was confined to areas of matrix formation (Plate 3.2 b and d). In cartilage, the enzyme was localized to cellular lacunae within the matrix whilst in bone like tissue a layer of AP positive cells was seen immediately adjacent to the bone surface. Whilst this is illustrated in Plate 3.2, detailed high power photomicrographs illustrating the AP distribution in sections from a chamber containing tissue from another colony, colony 67, are shown in Plate 4.9. The distribution of tissues formed within the chambers followed a similar pattern to that previously described for chambers cultured with suspensions of total marrow cells (Ashton et al, 1980; Mardon et al, 1987). Within the chambers the tissues were attached to the millipore filter and to the leucite ring. Typically the mineralizing, bone like tissue was adjacent to the millipore filter whilst the cartilaginous matrix was deeper, within the body of the chamber. The distribution and morphology of non-mineralized fibrous tissues were variable. Sometimes it was seen as a loose connective tissue matrix filling the whole of the chamber (Plate 3.3a). Often a dense connective tissue was seen lining the millipore filter (Plate 4.9e). Neither calcium deposition nor AP activity was found in the absence of osteogenic matrix, however, in the chamber containing tissue from Colony 39 (Plate 3.3), a tiny area of AP activity was found, but confined to less than 20 serial sections, making it about 50 μ m deep (Plate 3.3b).

Colony Number	AP	VK	Collagen Typing				Histological Observations
			I	II	III	V	
36	-	-	+	-	+	+	F.
39	+	-	+	-	+	+	F.
42	-	-	+	-	+	+	F.
44	-	-	+	-	+	+	F.
46	+	+	+	+	+	+	B.C.F.
56	+	+	+	+	+	+	B.C.F.
57	+	+	+	+	+	+	B.C.F.
58	-	-	+	-	+	+	F.

Fig 3.7 Summary of histological and immunocytochemical observations on sections from DC's, fixed in 95% alcohol after 2 months implantation in vivo. + indicates a positive staining reaction. AP is Alkaline Phosphatase activity and VK is the Von Kossa reaction. I,II,III and V refer to the results of immunocytochemical collagen typing. In the Histological Observation column, F is fibrous tissue, B is a bone like matrix and C is a cartilaginous matrix.

Immunocytochemical Collagen Typing.

Immunocytochemical staining was used to localize collagen types I, II, III and V. Types I, III and V were widely distributed in all chambers. Type II was confined to areas of skeletal tissue. There was no clear boundary between the areas resembling bone or cartilage which merged into each other, making it impossible to distinguish the two on the basis of collagen distribution. Plate 3.4 a, c and d shows the results of staining for type I, III and V collagens respectively from

a chamber containing tissue from colony 46, the colony illustrated in Plate 3.2. Collagen of all three types is distributed widely throughout the chamber in areas of mineralized and fibrous tissue. In mineralized areas type III staining occasionally appeared patchy (Plate 3.4c) suggesting some local loss of collagen type III. Collagen type II staining was confined to areas of mineralized matrix in marked contrast to adjacent fibrous tissue within the same chamber (Plate 3.4b).

In DC's where no bone or cartilage was formed there was no evidence of collagen type II staining, with diffuse type I, III and V staining throughout the fibrous connective tissue. This is illustrated in Plate 3.3 c to f which shows sections from the chamber containing cells from colony 39. As Plate 3.3 c to f are serial sections it can be seen that the AP positive nodule in Plate 3.3b is negative for collagen type II in Plate 3.3d.

3.3: DISCUSSION.

3.3a: GROWTH REQUIREMENTS OF STROMAL COLONIES IN VITRO.

Single marrow cell suspensions form discrete stromal colonies when explanted into live culture at seeding densities in the range 4×10^4 to 4×10^5 nucleated marrow cells cm^{-2} (Friedenstein, 1976; Castro-Malaspina et al, 1980; 1982). The conditions necessary for the growth of stromal colonies have not yet been

defined and FCS supplements of up to 20% were required. At lower seeding densities colony growth was not easily achieved whilst at higher densities a confluent monolayer was formed.

FCS supplements of 2% to 20% supported the formation of stromal colonies in primary marrow cell culture. In common with previous studies (Bjornson and McIntyre, 1986; Friedenstein, 1976), increasing the serum supplement to 20% increased the CFE. The reason for this remains unknown. One possibility may be that different subpopulations of stromal cells differ in their serum requirements. Some supporting evidence comes from a comparison of early and late calvarial derived cell populations released by sequential collagenase digestion (Wong et al, 1986). The former, resembling undifferentiated mesenchyme cells required high levels of serum (>10%) for initial growth and proliferation in vitro, whereas the latter osteoblast like cells attached in 2% serum. By analogy, it is possible to speculate that in the marrow stromal system, earlier progenitors required a larger serum supplement (>10%) for attachment and initial proliferation, whilst more differentiated precursors could adhere and proliferate in 2% serum.

Batches of serum varied considerably in their ability to support the growth of stromal colonies so comparison has only been made between experimental groups in medium supplemented with serum from the same batch. Even the best batches of FCS did not possess all the factors essential for the

proliferation of CFUf and additional factors were required to support the growth of marrow stromal precursors. These were provided by the non-adherent cells and it is suggested that the non-adherent cells which are the majority in primary marrow cultures, may act in the role of feeders in promoting stromal colony growth. The fall off in CFE with decreasing cell inoculum (Fig 3.1) is consistent with this role and confirms similar observations by Friedenstein (1990a; 1990b). Further evidence comes from the restoration of CFE to relatively high values for low cell inocula on addition of IRF (Fig 3.5) and its reduction when the non-adherent population was discarded (Fig 3.5).

3.3b: FACTORS PRODUCED BY BONE MARROW CELLS WHICH PROMOTE THE GROWTH OF STROMAL COLONIES.

The nature of the stimulus to proliferation of individual CFUf remains unknown, but it is likely that specific cytokines or growth factors produced by marrow cells are important. Many growth factors or cytokines have a mitogenic effect on a range of osteoblast like populations in vitro (reviewed by Canalis et al, 1989; Hauschka et al, 1988; Krane et al, 1988). A few studies have been directed towards the characterization of factors promoting the growth and differentiation of stromal precursors. Stromal colonies formed from a human marrow cell suspension in the presence of platelet rich plasma, but would not form in medium completed with platelet poor plasma (Bjornsen and McIntyre, 1986). CFE was restored in a dose

dependant manner by adding platelet derived growth factor (PDGF) to the platelet poor plasma.

EGF increased colony size but decreased AP expression (Owen et al, 1987), but, as EGF is not thought to be produced by haemopoietic cells (Fisher and Lakshmanan, 1990), it is unlikely to be the stimulus to growth of fibroblastic colonies provided by the IRF. Hc, in common with previous studies (Owen et al, 1987) promoted both colony number and AP expression suggesting a role in both stromal growth and differentiation.

When colonies were passaged and replated, populations grew to confluence in medium supplemented with 20% FCS in the absence of IRF. In this situation stromal cells were plated at a much higher density, $>10^4$ cells cm^{-2} . One possible explanation is that factors released by the stromal cells themselves were able to support stromal growth. It is well established that osteoblasts and periosteal tissue can synthesize a range of growth factors (Fig 1.3) but the role of these factors in interactions between stromal cells in vitro is unknown. In an analogous in vitro system there is evidence that specific cytokines released by one sub-population are mitogenic for another. Cell extracts of late released osteoblast-like cells from sequential collagenase digests of murine calvaria had a mitogenic effect on early released populations in vitro (Wong et al, 1987). This biological activity was localized by gel filtration to two peaks suggestive of the molecular weights

for the somatomedins (including IGF-1) or TGF- β . TGF- β (Canalis et al, 1985; 1989) and IGF-I (Wong et al, 1990) are both mitogenic for osteoblast-like cells and can be recovered from the medium during culture of these cells in vitro.

3.3c: TISSUE FORMED IN DC'S.

The tissue formed in diffusion chambers was either a fibrous tissue or a mixture of bone, cartilage and fibrous tissue. As in previous studies (Ashton et al, 1980), bone formed adjacent to the millipore filters whilst cartilage is found towards the centre. Culturing cells from marrow stromal colonies in DC's, surface area 154cm² and volume 0.015mm³ gave either bone or fibrous tissue. Chambers with a surface area of 79cm², volume 0.15mm³ gave either fibrous tissue or bone against the millipore filter with cartilage deep within the chamber (Friedenstien et al, 1987), thus, a nutrient or oxygen gradient within the chamber was an important determinant of phenotypic expression. Shaw and Basset (1967), demonstrated that phenotypic expression could be modulated by oxygen tension, and a low oxygen tension favoured cartilage formation with bone at a rather higher pO₂. In vitro, oxygen tension may affect AP expression used as a marker of osteoblastic differentiation in periosteal cells (Deren et al, 1990). Maximal AP expression was observed at oxygen tensions close to that in the capillaries, an observation that may well be of physiological significance.

After 60 days collagen types I, III and V were localized in all chambers although expression of each collagen type was more intense in bone like areas adjacent to the millipore filter. These results vary from those of Ashhurst et al (1990) and Mardon et al (1987) who sequentially observed tissues formed in DC's from a primary marrow cell suspension up to 64 days after implantation. Fibrous tissue formed initially and was characterized by collagen types I, III, V, laminin and fibronectin, giving way to types I and V in areas of bone formation.

These differences cannot be explained but could be related to the composition of the different cell populations implanted in each experimental situation. A marrow cell suspension is composed mostly of haemopoietic cells with a small number of stromal precursors. In DC's cells of the haemopoietic lineage do not survive leaving the stromal precursors to re-populate the chambers de-novo (Bab et al, 1986). Friedenstein (1987) demonstrated that stromal precursors develop at different rates and only 30% of those tested had osteogenic potential, a result confirmed by the present study. Thus, tissue formed in chambers implanted with marrow cell suspensions is formed by a few precursors which differ in their differentiation potential. By comparison, when implanting cells from single colonies about 5×10^5 stromal cells from a single precursor were placed in each DC.

Factors produced by stromal cells may contribute to the loss of type III collagen in areas of osteogenesis. Osteoblast like cells in vitro produce collagenase (Sellers et al, 1978; Sakamoto and Sakamoto, 1982; Meikle, 1976). It is not known if different clonal marrow stromal populations differ in their capacity to produce collagenase but it is interesting to speculate that cells with the capacity to produce collagenase may be present in DC's derived from marrow cell suspensions but not in those from single clones.

Type II collagen was localized to areas resembling skeletal tissues but, like Ashhurst et al (1990) and Mardon et al (1987), cartilage and bone were not completely discrete tissues and cartilage and bony areas overlapped and merged into each other. These areas were considered analogous to chondroid bone which has been described in vivo.

Using in vitro tissue culture techniques and an in vivo DC assay, it has been possible to confirm that, in marrow, there is a small population of stromal stem cells with the potential to differentiate into bone, cartilage and fibrous tissue.

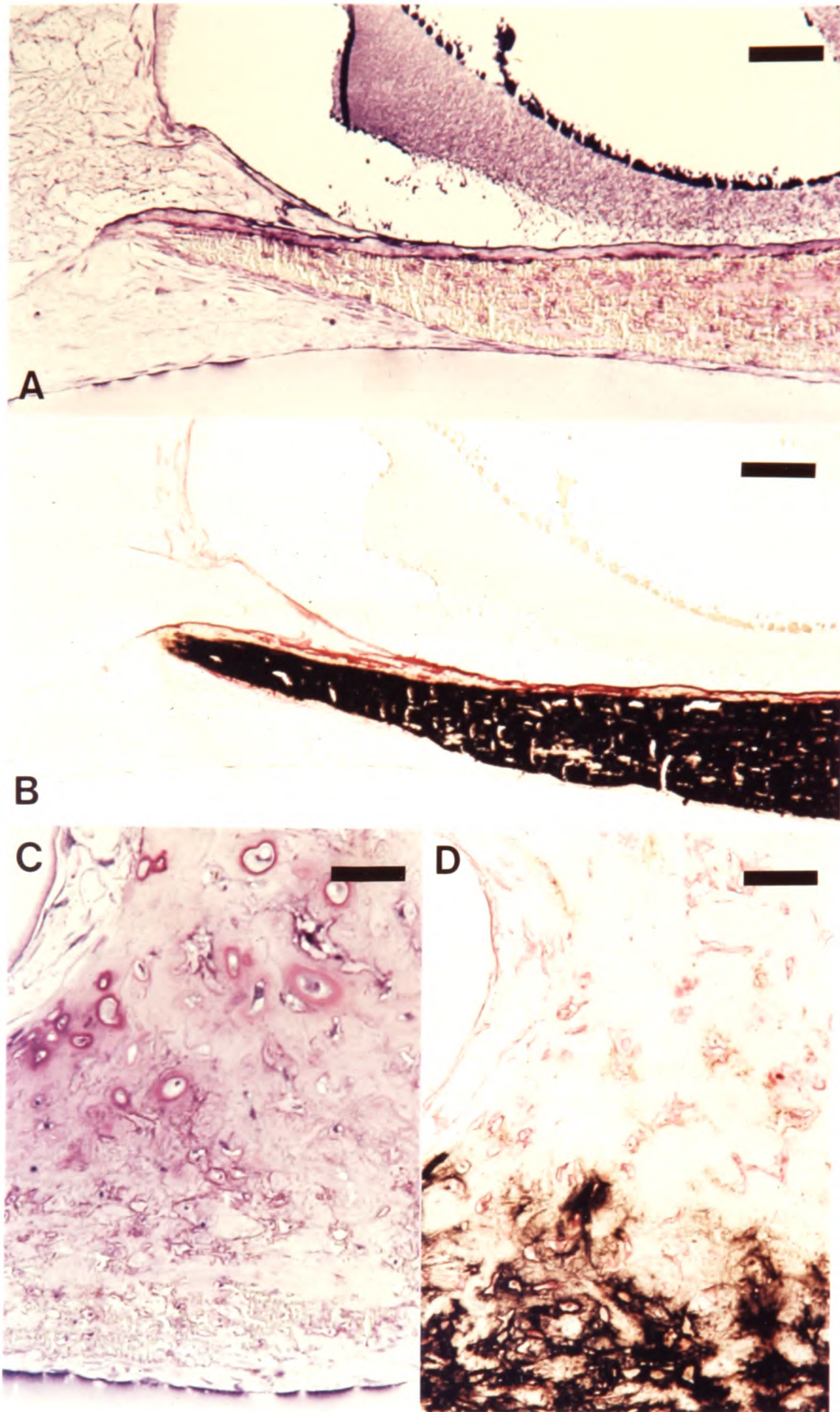


Plate 3.2.

Photomicrograph showing 5 μ m sections of a DC containing cells from colony 46, a fibroblastic colony which formed a mineralized matrix on implantation in vivo. A and C are stained with toluidine blue. B and D are stained to localize AP activity and counterstained with Von Kossa. Bar is 100 μ m.

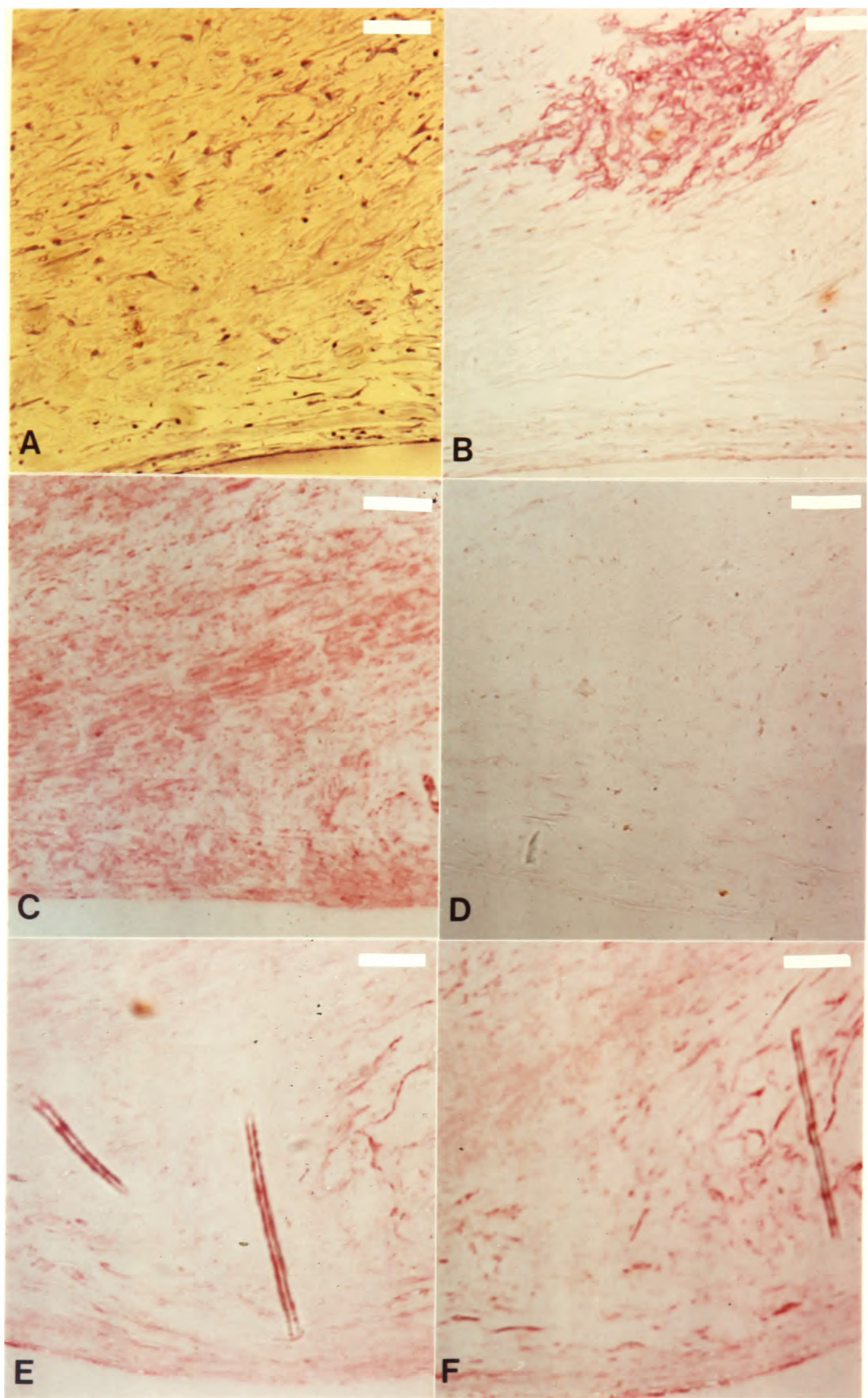


Plate 3.3.

5 μ sections of a DC containing tissue from colony 39 where no mineralized matrix was formed. A is stained with toluidine blue and B to localize AP activity. In B a small, localized area of AP activity can be seen.

C, E and F show diffuse reaction throughout the fibrous tissue when staining with a polyclonal antiserum to collagen types I, III and V respectively. D shows a section stained for collagen type II which showed no reaction. Bar is 100 μ m.

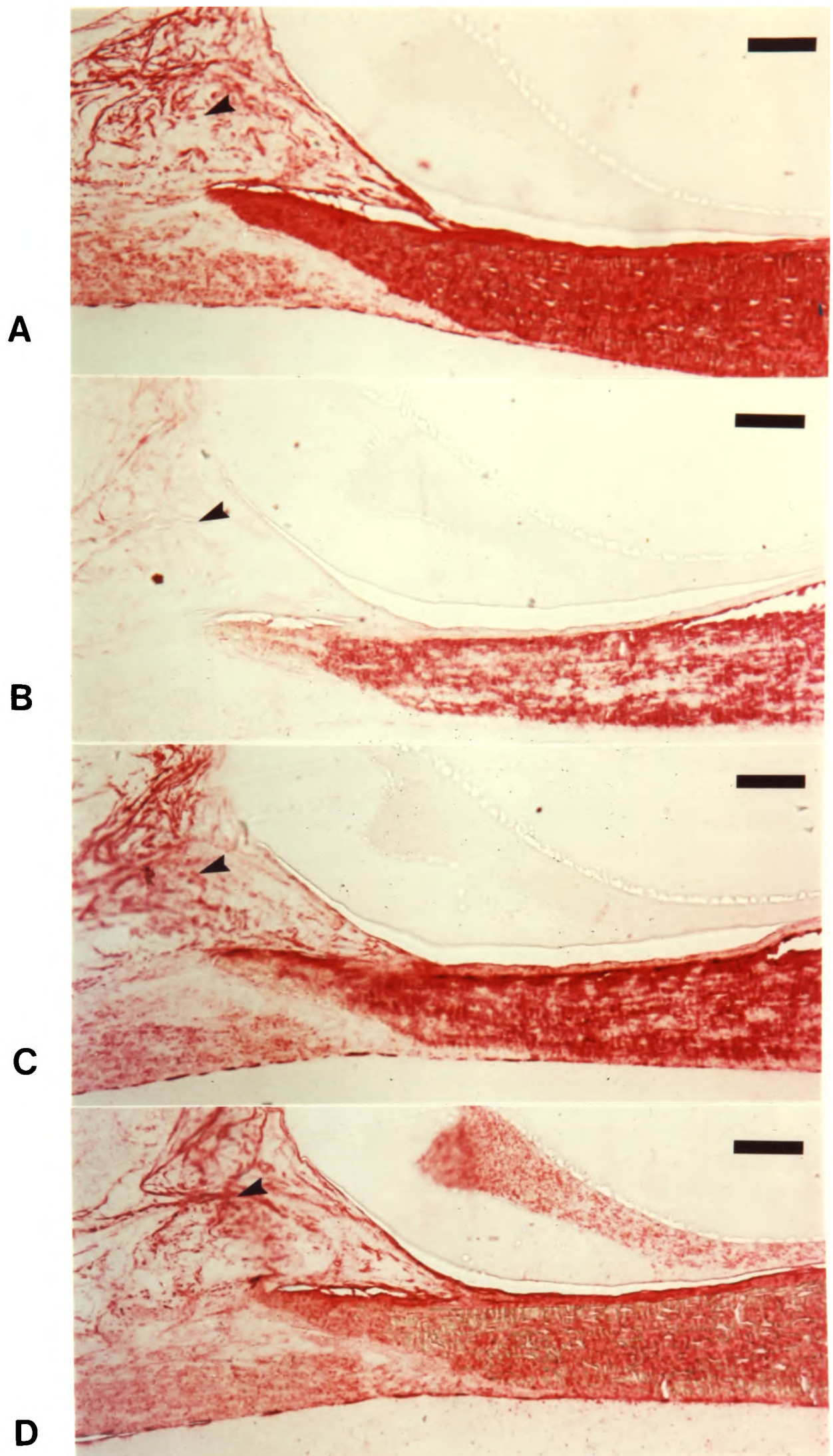


Plate 3.4.

Photomicrographs of 5 μ m sections from a DC containing cells derived from colony 46, a fibroblastic colony which formed a mineralized matrix on implantation in vivo. Sections are stained to localize A; Collagen type I, B; Collagen type II, C; Collagen type III, and D; Collagen type V. A particular point to note is that the fibrous tissue stains for collagen types I, III and V but not II (arrowed). Areas of mineralized matrix stain for collagen type I, II, III and V. Bar is 100 μ m.

CHAPTER 4.ADIPOCYTIC COLONIES CULTURED FROM MARROW
CELLS.4.1: INTRODUCTION.

In the previous chapter colonies with a typical fibroblastic morphology were cultured in vitro. In vivo assay of individual colonies in DC's showed that about 30% had osteogenic potential, which is in agreement with previous findings (Friedenstein, 1987). A similar analysis, however, has not yet been applied to colonies expressing other stromal phenotypes in vitro. Such studies are of great interest as they provide data on the pluripotential nature of the putative stromal stem cell (chapter 1).

To investigate these questions a new approach was adopted. By replacing medium supplemented with 20% FCS with that supplemented with 15% autologous rabbit plasma (RbP) conditions were identified that promoted differentiation in an adipocytic direction. Subsequently, adipocytic colonies were expanded and assayed in DC's.

The adipocytic phenotype is well suited to the investigation of stromal cell differentiation in vitro. Cells at different stages of differentiation within colonies can be recognised on well established morphological grounds. Undifferentiated adipocytes are fibroblastic. Pre-adipocytes have a characteristic rounded appearance with a granular cytoplasm,

whilst mature adipocytes have large, refractile, fat filled vacuoles filling the cytoplasm (Van and Roncari, 1982; Roncari and Van, 1978; Cormack, 1989; Johnson and Greenwood, 1988). Furthermore, because differentiating adipocytes can be characterized on the basis of morphology, individual colonies were classified on the basis of the number of cells and the proportion of differentiated adipocytes present. On the assumption that stem cells gave rise to larger colonies with a range of cell types, and later progenitors to smaller colonies with a few differentiated cells, evidence is presented here to show that there are a range of adipocytic precursors present in marrow extending from earlier stem cells to later committed progenitors.

4.2: RESULTS.

4.2a: COMPARISON OF CFE IN FCS OR AUTOLOGOUS RABBIT PLASMA (RBP).

When single cell suspensions of 10^6 - 3×10^6 primary marrow cells were plated into flasks in medium with 15% RbP $\pm 10^{-7}$ M Hc, discrete stromal colonies were observed after 7 to 10 days in culture, compared with 14 to 21 days in medium with 20% FCS $\pm 10^{-7}$ M Hc (Chapter 3). In two experiments out of four comparing the CFE with 15% RbP and 20% FCS, the CFE was greater with 15% RbP than 20% FCS whilst in the other two there was no difference (Fig 4.1 (i) and (ii)). When allowed to attach in BGJb medium without serum for 2 hours, the non adherent cells

removed and the adherent cells washed before adding medium completed with 15% RbP and Hc or 20% FCS and Hc (Fig 4.1 (iii) and (iv)), the number of colonies formed with FCS was reduced by a factor of 10, whilst with RbP the reduction was about 30%. Furthermore, removal of non adherent cells made serial observations easier.

4.2b: MORPHOLOGY OF ADIPOCYTIC COLONIES.

Colonies grown in RbP had a characteristic adipocytic morphology. Typically, they were smaller in diameter than those grown in medium supplemented with FCS, and by 14 days ranged from 0.2mm to 3.0mm in diameter. Initially, pre-adipocytic cells could be identified by an intensely granular cytoplasm. Then tiny cytoplasmic vacuoles could be seen which coalesced to form large, lipid filled structures filling the cytoplasm and obscuring most of the anatomical features of the cell (Plates 4.1b, 4.2, 4.3b). On the basis of observations made between days 11 and 14 in vitro, colonies were placed into one of three groups labelled types I, II or III respectively. The difference in the timing of these observations reflects subjective differences in the rate at which cultures developed between experimental animals.

Type I colonies were small, often less than 0.4mm diameter (Plate 4.1b) consisting of a few large cells, the majority of which were characterized by extremely dense cytoplasmic accumulations of lipid. Occasionally, large flattened

fibroblastic cells were seen with little or no lipid. In many colonies a cell count was possible and there were rarely more than 50 cells present.

Type II colonies represented a wide ranging group up to 1.5mm in diameter, intermediate in morphological characteristics between types I and III, and consisting mainly of large adipocytic or fibroblastic cells with a few of the small, granular polygonal type.

A typical type II colony, 1.1mm in diameter, cultured in BGJb, 15% RbP and 10^{-7} M Hc is shown in Plate 4.2. Whilst there are many large adipocytic cells present, some cells retain a granular, pre-adipocytic morphology. Furthermore, the colony has a much greater diameter to the type I colony illustrated in Plate 4.1b.

Type III colonies were characterised by a greater range of cell types than the type I colonies. Whereas type I colonies were almost entirely adipocytic, a range was seen in type III colonies including large, flattened fibroblastic cells, small round or polygonal pre-adipocytic cells with a granular cytoplasm to adipocytes with refractile, lipid filled cytoplasmic vacuoles.

A type III colony with a diameter of 3.1mm on day 11 of culture is shown in Plate 4.3b and c. Peripherally, fibroblastic cells, and pre-adipocytic cells with a granular

cytoplasm were seen (Plate 4.3c). Towards the centre (Plate 4.3b) cells were almost exclusively adipocytic and cellular morphology was largely obscured by the extensive, refractile lipid accumulations.

Type III colonies varied widely in the proportion of adipocytes present. Plate 4.4a and b shows two colonies cultured in BGJb and 15% RbP, fixed in formol calcium and stained with Oil-Red-O to localize lipid. Plate 4.4b shows a colony with a large number of adipocytes centrally whilst that in Plate 4.4a had very few adipocytes and these were widely dispersed throughout the colony.

Individual type III colonies were too small for isolation and counting, and were incapable of further growth without modification of the culture conditions (chapter 4.2g), thus cell counts were not possible. Nevertheless, comparison of a type I colony (Plate 4.1b) and a type III colony (Plate 4.3 b and c) suggest that the number of cells present in the type III colony is an order of magnitude greater than that in the type I.

4.2c: SEQUENTIAL OBSERVATIONS ON COLONY TYPES I, II AND III.

Sequential observations were carried out on individual type I, II and III colonies at two time points 4 days apart, either on days 7 and 11 or days 10 and 14 of culture. Type I colonies

changed little in size and morphology between the 1st and 2nd observation (Plates 4.1a and b). Type II colonies were intermediate between types I and III. Type III colonies frequently appeared at the first observation as tightly packed, polygonal clusters of cells characterized by a granular cytoplasm with occasional, small cytoplasmic fat vacuoles. Within 4 days colonies had grown considerably, the amount of adipogenesis had increased progressively with areas of adipogenesis centrally (Plate 4.3 a and b). The degree of adipogenesis varied widely and was recorded using the subjective scoring system outlined below;

SCORE	OBSERVATION.
0	No adipocytic cells
+	Occasional solitary fat cells. <10/x100 field.
++	Well spaced fat cells separated by other cell types >10/x100 field. An example is shown in <u>Plate 4.4c</u> .
+++	Adipocytic cells are widespread throughout the colony but clumps of fat cells are still separated by other cell types. An example is shown in <u>Plate 4.4d</u> .
++++	The central area of the colony is wholly adipocytic obscuring the underlying cell morphology. Peripherally there may be a small band of granular or fibroblastic cells. <u>Plates 4.1 and 4.3</u> illustrate +++++ colonies.

Fig 4.2 shows the number of such colonies formed together with the number adipogenic at the +/++ levels or the +++/++++ levels. Fig 4.3 gives the results of three experiments

measuring the mean diameter of colony types I, II and III cultured in BGJb with 15% RbP with Hc (Fig 4.3 column (i)) or without Hc (Fig 4.3 column (ii)) on day 10 and shows a consistent and statistically significant difference ($p \leq 0.01$) between the mean diameters of each colony type. In Fig 4.4a the mean diameters of colony types I, II and III are shown at the 1st and 2nd serial observations for two representative experiments. There was no significant increase in diameter between serial observations on colony types I and II, however, there was a significant difference between the diameter of the type III colonies at the two time points ($p \leq 0.01$). Again, in these experiments colonies cultured in BGJb medium with or without Hc were compared.

This data does not show the fate of individual colonies classified as types I, II and III at the first serial observation. This has been investigated and the results are shown in Fig 4.4b. For an experiment in which colonies were cultured in BGJb medium with 15% RbP, Fig 4.4b (i) shows the numbers of each colony type at the first serial observation, and Fig 4.4b (ii) the classification of the same colonies 4 days later. For example, of 7 type I colonies recorded on day 10, six were type I when observed on day 14 whilst 1 had assumed the morphology of a type II colony. Fig 4.4b (iii) and (iv) show similar results from the same experiment for colonies cultured in BGJb medium, 15% RbP and Hc. Fig 4.4b (v) to (viii) give the results for a repeat experiment in which the sequential observations were made on days 7 and 10.

Generally, colonies retained their original classification between serial observations. Out of a total of 211 colonies, 87% remained in their original classification after 4 days (Fig 4.4b).

4.2d: OBSERVATIONS ON COLONY TYPES I, II AND III IN THE PRESENCE OF HYDROCORTISONE (Hc).

Fig 4.3 (i) and (ii) compare the number of each type of colony and the mean diameter with or without Hc. No significant difference was found when comparing the diameters of each group. There were, however, differences in the distribution of colonies between the groups. In the absence of Hc, 12% of colonies were type I (Fig 4.3(i)), doubling to 23% when Hc was added to the culture medium (Fig 4.3(ii)). Conversely, the percentage of type III colonies dropped from 74% to 60% in the presence of Hc. Fig 4.4a and Fig 4.4b compare serial observations on colonies grown in medium with or without Hc and show no difference in the growth characteristics of individual colony types. Again, however, Hc in the culture medium caused a shift in the distribution of colonies in favour of an increased percentage of type I colonies (Fig 4.4b). In Fig 4.2 the number of colonies, the number of adipogenic colonies and the number adipogenic at the +++/++++ level were compared with or without Hc. Whilst the number formed was similar in both groups, the number of adipogenic colonies and the intensity of adipogenesis was greater in the presence of the glucocorticoid.

4.2e: SUMMARY OF OBSERVATIONS ON COLONIES CULTURED IN
RABBIT PLASMA.

1. Colonies have been classified according to size and morphology into three categories.
2. Type III colonies are the largest with the most growth potential. Type I colonies are the smallest with the least growth potential and consist of, almost exclusively, differentiated adipocytes. Type II colonies fall between types I and III.
3. The majority of colonies in control cultures are type III (>70%).
4. The presence of 10^{-7} M Hc had little effect on colony size but increased the proportion of type I at the expense of type III colonies. In other words Hc stimulated adipocytic differentiation at the colony level.

Exp't.	Inoc'n	No 2hr Pre-incubation.		With 2hr Pre-incubation	
		Column (i) BGJb+20%FCS +Hc.	Column (ii) BGJb+15%RbP +Hc.	Column (iii) BGJb+20%FCS +Hc.	Column (iv) BGJb+15%RbP +Hc.
17.11.87	2.8	0.79(0.05)	0.51(0.09)	0.11(0.03)	0.33(0.06)
17.12.87	7.2	0.64(0.07)	0.62(0.08)	0.05(0.02)	0.39(0.04)
5.8.88	6.0	0.43(0.07)	1.32(0.14)	0.04(0.02)	0.99(0.03)
	3.0	0.45(0.06)	0.92(0.07)	0	0.64(0.14)
5.6.89	3.0	1.35(0.25)	3.66(0.21)	0	3.08(0.2)

Fig 4.1 Comparison of the Number of Colonies Formed in BGJb medium and 10⁻⁷M Hc with 20% FCS or 15% RbP for four experiments.
 Inoc'n is the primary nucleated marrow cell inoculum per 25cm² flask x10⁶.
 The number of colonies formed is expressed as CFE x10⁻⁵ (Mean(SD)).

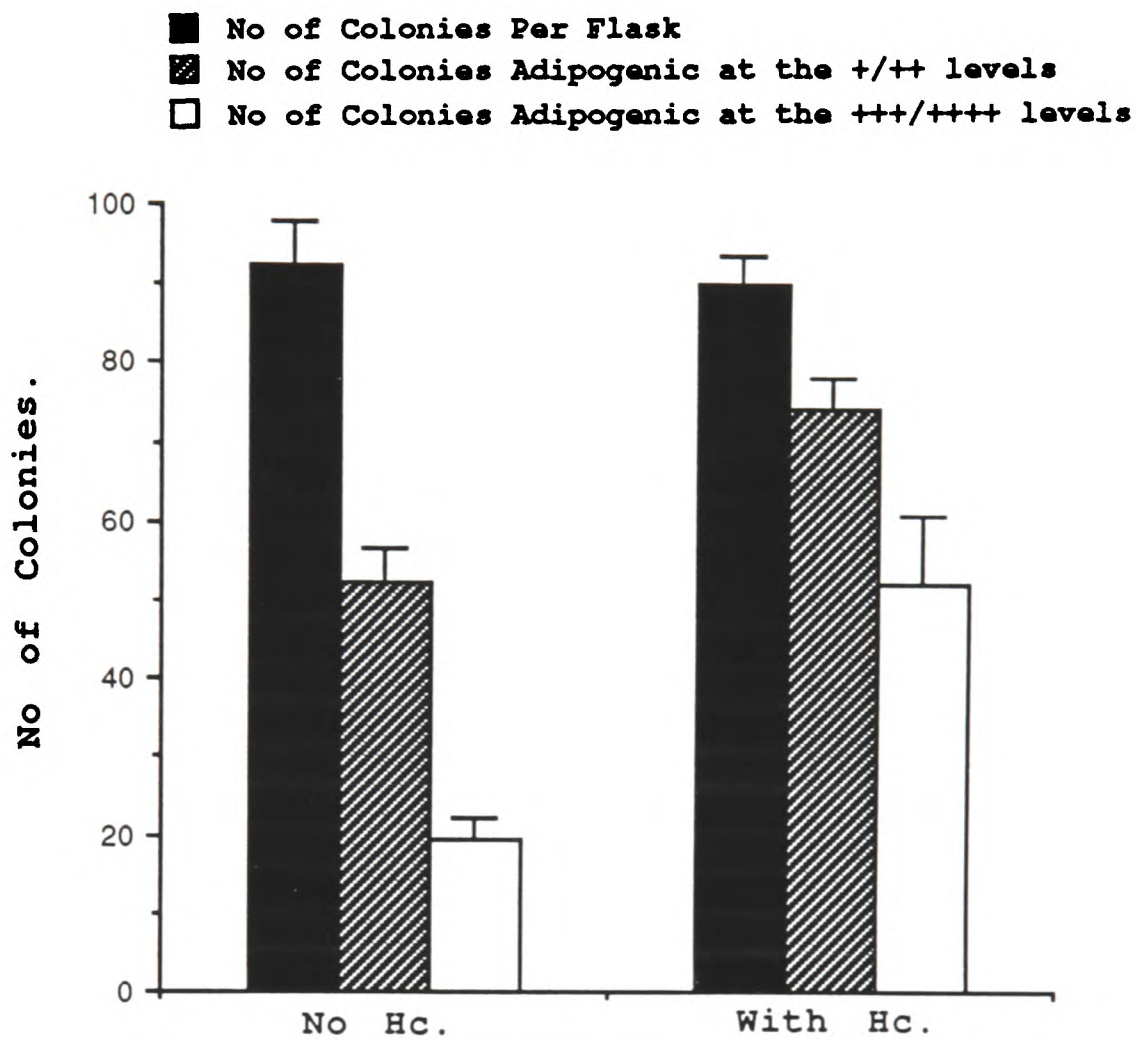


Fig 4.2.

Results of a representative experiment comparing the number of colonies per flask, the number of colonies adipogenic at the +/- levels and the number of colonies adipogenic at the +++/++++ levels when cultured for 14 days in BGJb and 15% RbP ±Hc. No of flasks per group = 4.

Exp't	Colony Type					
22.12.88 5.6.89 9.2.90 n	No Hydrocortisone			With Hydrocortisone		
	I	II	III	I	II	III
	0.41(0.29)	0.8(0.42)	1.14(0.39)	0.4(0.22)	0.92(0.26)	1.43(0.47)
	0.4(0.22)	0.8(0.35)	1.36(0.53)	0.52(0.27)	0.76(0.17)	1.31(0.47)
	0.48(0.25)	0.7(0.20)	1.08(0.32)	0.33(0.14)	0.68(0.19)	1.32(0.40)
	22 (12%)	28 (14%)	140 (74%)	49 (23%)	36 (18%)	125 (60%)

Fig 4.3

Table to show the diameter, mean(SD), of colony types I, II and III cultured in BGJb, 15% RbP $\pm 10^{-7}$ M Hc. The results for three experiments are given. n is the number of colonies counted.

A		NO HYDROCORTISONE			WITH HYDROCORTISONE		
COLONY TYPE		TYPE I	TYPE II	TYPE III	TYPE I	TYPE II	TYPE III
EXP'T 10.2.89							
DAY 10		0.23(0.22)	0.77(0.35)	0.65(0.23)	0.4(0.2)	0.86(0.22)	0.76(0.2)
DAY 14		0.25(0.1)	0.9(0.14)	1.35(0.35)	0.4(0.23)	0.98(0.15)	1.52(0.46)
EXP'T 5.6.89							
DAY 7		0.36(0.27)	0.7(0.35)	0.67(0.27)	0.34(0.2)	0.54(0.2)	0.58(0.25)
DAY 11		0.4(0.22)	0.8	1.36(0.53)	0.52(0.27)	0.76(.17)	1.31(0.47)

B		NO HYDROCORTISONE		WITH HYDROCORTISONE	
EXP'T 10.2.89		DAY 10(i)	DAY 14(ii)	DAY 10(iii)	DAY 14(iv)
		TYPE I (n=7)	TYPE I (n=6) TYPE II (n=1) TYPE III (n=0)	TYPE I (n=23)	TYPE I (n=18) TYPE II (n=4) TYPE III (n=1)
		TYPE II (n=3)	TYPE I (n=0) TYPE II (n=1) TYPE III (n=2)	TYPE II (n=12)	TYPE I (n=0) TYPE II (n=5) TYPE III (n=7)
		TYPE III (n=30)	TYPE I (n=0) TYPE II (n=0) TYPE III (n=30)	TYPE III (n=16)	TYPE I (n=0) TYPE II (n=0) TYPE III (n=16)
EXP'T 5.6.89		DAY 7(i)	DAY 11(ii)	DAY 7(iii)	DAY 11(iv)
		TYPE I (n=6)	TYPE I (n=4) TYPE II (n=2) TYPE III (n=0)	TYPE I (n=15)	TYPE I (n=13) TYPE II (n=2) TYPE III (n=0)
		TYPE II (n=3)	TYPE I (n=0) TYPE II (n=1) TYPE III (n=2)	TYPE II (n=9)	TYPE I (n=0) TYPE II (n=6) TYPE III (n=3)
		TYPE III (n=47)	TYPE I (n=0) TYPE II (n=0) TYPE III (n=47)	TYPE III (n=40)	TYPE I (n=0) TYPE II (n=3) TYPE III (n=37)

Fig 4.4

A: The diameter (mm), mean(SD), and colony type of each colony present was sequentially recorded at 3 day intervals in two exp'ts. Colonies were cultured in BGJb with RbP ±Hc.

B: For exp't 10.2.89, the no of colonies of each type, cultured in the presence (iii) or absence (i) of Hc was recorded at the first observation. The same colonies were observed at the second time point and the classification into colony type repeated (ii) and (iv). For example, of 7 type I colonies observed on day 10 (i), 6 were still type I on day 14 (ii), thus colonies generally retained their initial classification. Similar results are given for exp't 5.6.89. (n=no of colonies).

4.2f: AUTORADIOGRAPHY.

Colonies observed on day 14 were photographed in vitro before pulse labelling with ^3H Thymidine. Following fixation and histochemical localization of alkaline phosphatase (AP) activity, the distribution of cells in S phase was determined by autoradiography. Plate 4.5 a and b show a representative type I colony on day 14 of culture and the same colony following fixation, AP localization and autoradiography. No evidence of ^3H thymidine uptake or AP activity was seen in type I colonies. In type III colonies, adipogenesis and AP activity was seen within the same colony, however, because of the disruption of adipocytic cells caused by the 95% alcohol fixation required for AP histochemistry, it was not possible to ascertain whether individual cells were both adipogenic and had AP activity. AP activity or adipogenesis was either widely distributed throughout the colony or localized to a central region (Plate 4.6 a, b and c). In most type III colonies ^3H thymidine uptake was confined to cells towards the periphery of the colony, but examples were occasionally seen where ^3H thymidine uptake was more widely spread throughout the colony. These colonies were characterized by a larger proportion of the tightly packed, polygonal, granular cells, or cells with tiny cytoplasmic fat vacuoles rather than cells which were densely adipocytic or AP positive.

4.2g: THE EFFECT ON ADIPOCYTIC COLONIES OF A CHANGE FROM RbP TO FCS.

Well separated colonies were selected in flasks initiated in 15% RbP with 10^{-7} M Hc, and observed on day 8. Medium in these flasks was changed to that completed with 20% FCS with Hc, whilst the remainder were continued in the original medium. Colonies continued in 15% RbP and Hc maintained an adipocytic morphology, and the mean diameter increased x1.9 from 0.63 ± 0.25 mm to 1.21 ± 0.91 mm (Fig 4.5) Those transferred to FCS, however, rapidly lost their adipocytic morphology, assuming fibroblastic characteristics, and by day 12, cytoplasmic fat vacuoles were almost entirely absent from the cultures. These colonies were larger, reflecting a x5.3 increase in diameter from 0.72 ± 0.3 mm to 3.42 ± 1.32 mm (Fig 4.5). This loss of adipocytic characteristics and increase in diameter was a feature of all colony types, although type I colonies did not appear to have as great a potential for increased growth as type III colonies. Plates 4.7 a and b and 4.8 a, b and c show types I and III colonies respectively, observed on day 8, before the change to medium completed with 20% FCS. Plates 4.1 a and b and 4.3 a, b and c show similar colonies maintained in 15% RbP.

CONDITIONS.	NO OF COLONIES.	DIAMETER		% ADIPOGENIC	
		DAY 7.	DAY 13.	DAY 7.	DAY 13.
13 DAYS IN 15% RbP	25	0.63 (0.25)	1.2 (0.73)	73%	100%
15% RbP CHANGING ON DAY 7 TO 20% FCS.	27	0.72 (0.3)	3.4 (1.34)	61%	3.7%

Fig 4.5

Results from a representative experiment in which the diameter in mm, mean(SD), and adipogenesis of colonies cultured for 13 days in BGJb medium, 15% RbP and 10⁻⁷M Hc was compared to those in which the medium was changed to that supplemented with 20% FCS on day 7.

4.2h: IN VIVO ASSAY OF ADIPOGENIC COLONIES.

Adipocytic colonies seldom grew to more than 3.0mm diameter in RbP and did not survive direct isolation by ring cloning. After observation on day 10, therefore, the medium was changed to that supplemented with FCS and 10^{-7} M Hc, and under these conditions adipocytic colonies grew to diameters large enough for successful isolation by ring cloning. After 19 to 24 days in primary culture, well separated colonies which were at the larger end of the spectrum of colony sizes were isolated for expansion and implantation.

	Implanted.	Discarded.
No of Colonies.	8	13
Diam (mm) at time of isolation.	5.7(1.3)	4.8(0.9)
No. of Cells at time of isolation.	3.7(3.1)*	1.0(0.8)*
No. of days to isolation.	19.7(3.3)	23.4(4.0)
No. of passages before being implanted /discarded.	5-6	1-5
No. of Cells inoculated per DC.	15.4(6.2)	----

Fig 4.6 Data for adipocytic colonies isolated by cloning rings and expanded for implantation in vivo in DC's. Results are given as a Mean(SD) or as a range. The number of cells at the time of isolation and the number inoculated into DC's are given x 10⁵.
* p ≤ 0.05

Data for 21 adipocytic colonies isolated is given in Fig 4.6. Eight were subsequently implanted in vivo in DC's whilst the remainder failed to give a sufficient number of cells for implantation and were discarded. Fig 4.7 summarizes histological observations made on the chambers following fixation. Three formed a mineralizing matrix resembling bone and cartilage together with fibrous tissue, two formed fibrous tissue alone, two were infected and one chamber contained no tissue at all. As with tissue from fibroblastic colonies (Chapter 3) mineral and AP activity was only seen in chambers

in which bone and cartilage formed. Plate 4.9 a shows an adipocytic colony, Colony 67, on day 10 before changing to medium supplemented with FCS, isolation, expansion and implantation. Plates 4.9 b, c, d and e are sections of the DC containing tissue from Colony 67. Bone is formed close to the millipore filter. Between the filter and the mineralizing tissue is a cell layer rich in AP activity. This is separated from the mineralizing bone by an acellular 'osteoid' layer. Deep to the bone is a cartilage like matrix.

Immunohistochemical collagen typing was performed on serial sections from selected chambers and gave a similar pattern to that described for fibroblastic colonies in chapter 3.

Collagen types I, III and V were found in each chamber whilst type II was restricted to areas of skeletal matrix formation.

Colony Number	AP	VK	Histological Observations
65	-	-	F.
66	-	-	Nothing
67	+	+	B.C.F.
68	+	+	B.C.F.
72	-	-	F.
76	-	-	Infected
77	-	-	Infected
78	+	+	B.C.F.

Fig 4.7 Summary of histological and immunocytochemical observations on sections from DC's, fixed in 95% alcohol after 2 months implantation in vivo. + indicates a positive staining reaction. AP is alkaline phosphatase activity and VK is the Von Kossa reaction. F is fibrous tissue, B is a bone like matrix and C is a cartilaginous matrix.

4.3: DISCUSSION.

4.3a: MORPHOLOGY OF COLONIES CULTURED IN RBP.

Autologous RbP promoted the growth of rabbit marrow stromal colonies consisting almost entirely of cells with a pre-adipocytic or adipocytic morphology. Other studies have demonstrated adipogenesis in clonal populations of murine marrow stromal cells in the presence of horse serum and Hc (Kodama et al, 1983), FCS and Hc (Greenburger, 1978), and of human marrow stromal cells in the presence of FCS and Hc (Greenburger, 1979) or human plasma and Hc (McIntyre and Bjornson, 1986). In contrast to the present study, the adipocytic colonies contained a range of cells extending from those with a fibroblastic morphology to differentiated adipocytes. It is not known if these are species differences or secondary to differences in culture conditions. The factors in RbP which stimulate adipocytic differentiation have not yet been determined, however, there is evidence that a distinct serum factor of 58-68KDa in rat serum promotes adipogenic conversion in primary cultures of extra-medullary rat adipocytes (Li et al, 1989). Similar factors have not yet been sought in rabbit serum.

4.3b: PRECURSORS OF COLONY TYPES I, II AND III.

The experiments described here divide colonies cultured in medium supplemented with RbP into three groups on the basis of

their capacity for growth and differentiation in vitro. It is suggested that type I colonies are the progeny of precursors which, under these conditions, are restricted in their proliferative and differentiation potential. Type III colonies with their greater cell number, and with a greater range of cell types present, are derived from an earlier precursor with stem cell characteristics. Type II colonies with characteristics mid way between types I and III probably reflect precursors in the middle of the range. Analogous results have been reported for in vitro studies in other systems, for example epidermal keratinocytes (Barrandon and Green, 1987) or cells of the haemopoietic lineages (Metcalfe, 1988), but this is believed to be the first application of clonal analysis to the study of marrow stromal adipocytes in vitro.

4.3c: TYPE III COLONIES.

The autoradiographic studies demonstrated that adipogenic type III colonies took up ³H-thymidine and expressed AP activity to varying degrees. This indicated that, in some adipogenic colonies, some cells were actively dividing and in others, a proportion of cells were capable of expressing AP activity. The precursors of these type III colonies, therefore, had proliferative potential and multipotentiality, both of which are stem cell characteristics.

Growing cells were located towards the periphery of colonies, where cells tended to have a pre-adipocytic morphology rather than that of differentiated adipocytes, which were found towards the centre. Loss of proliferative potential, appeared to be associated with acquisition of a differentiated phenotype. These results were consistent with observations on marrow stromal colonies where differentiated cells were found centrally, and less differentiated cells retaining some proliferative capacity, peripherally (Owen et al, 1987; Owen and Friedenstein, 1988; Lanotte et al, 1982).

There was considerable heterogeneity between type III colonies which varied in size, the proportion of adipogenic cells and expression of AP activity, indeed, no two colonies appeared identical. This observation is consistent with current concepts of stromal precursor cells (Owen 1987, Aubin et al, 1990)(chapters 1.2 and 1.3). Each precursor is thought to lie at a different position along a differentiation pathway ranging from a multipotential stem cell to a late committed progenitor. Progression along the pathway is associated with loss of proliferative and differentiation potential. On this basis it can be argued that the precursors of type III colonies are at different stages of differentiation close to the stem cell end of the differentiation pathway, thus, each has a slightly different differentiation and proliferative potential.

Further support for this argument comes from studies where comparison has been made between series of stromal clones, some of which have osteoblast-like characteristics (Aubin et al, 1982; Guenther et al, 1989). A common finding was that clones were not identical, differing widely in their growth potential, expression of many features associated with the osteoblast phenotype including cyclic AMP response to PTH, collagen synthesis, synthesis of bone specific proteins such as osteocalcin and expression of AP activity. These observations may be explained in terms of the cells of origin of the clones being at different positions along the differentiation pathway, hence they had different differentiation and proliferative potentials.

4.3d: TYPE I COLONIES.

In contrast to type III colonies, type I colonies were small, and consisted almost wholly of differentiated adipocytes. They did not take up ³H-thymidine nor express AP activity. They had little proliferative potential when cultured in BGJb medium supplemented with 15% RbP and did not increase in size between sequential observations at three day intervals in live culture. These results support the conclusion that precursors of type I colonies were derived from late stage precursors or committed progenitors.

4.3e: OSTEOGENIC POTENTIAL OF ADIPOCYTIC CELLS.

There is evidence to suggest that marrow osteoblasts and adipocytes share a common precursor. On implanting single colonies in vivo under the renal capsule a complete microenvironment capable of supporting haemopoiesis was formed including bone and fat (Friedenstein, 1980). The results presented here confirm this observation.

A question of considerable relevance to the study of the stromal lineage is how far cells can differentiate in an adipocytic direction before reverting to a more proliferative stage and differentiating in an osteoblastic direction. The adipocytic colonies, cultured in RbP + Hc, became progressively loaded with lipid and then stopped growing. In order to re-initiate growth, it was necessary to change the serum supplement to FCS. Under these conditions, all cells in the adipocytic colonies lost their lipid, reverted to a fibroblastic morphology and were capable of further growth. Colonies could then be isolated and expanded to give enough cells for implantation. Furthermore, the growth kinetics of the fibroblastic populations produced from the adipocytic colonies argued in favour of the majority of cells being capable of reversion to a fibroblastic morphology and subsequent growth. Some support for this observation comes from previous work on murine marrow stromal adipocytes (Lanotte et al, 1982) and on cloned populations of pre-adipocytes from the 3T3-L1 cell line (Hoerl and Scott, 1989),

which showed that even relatively mature adipocytes retained some proliferative capacity.

It was clear from these results that cells retain proliferative and osteogenic potential even though some degree of adipocytic differentiation may have taken place. It was possible to conclude that divergence of the osteoblast and adipocyte lineages must occur relatively late in the stromal lineage. Some clinical implications of these results are considered further in chapter 7.

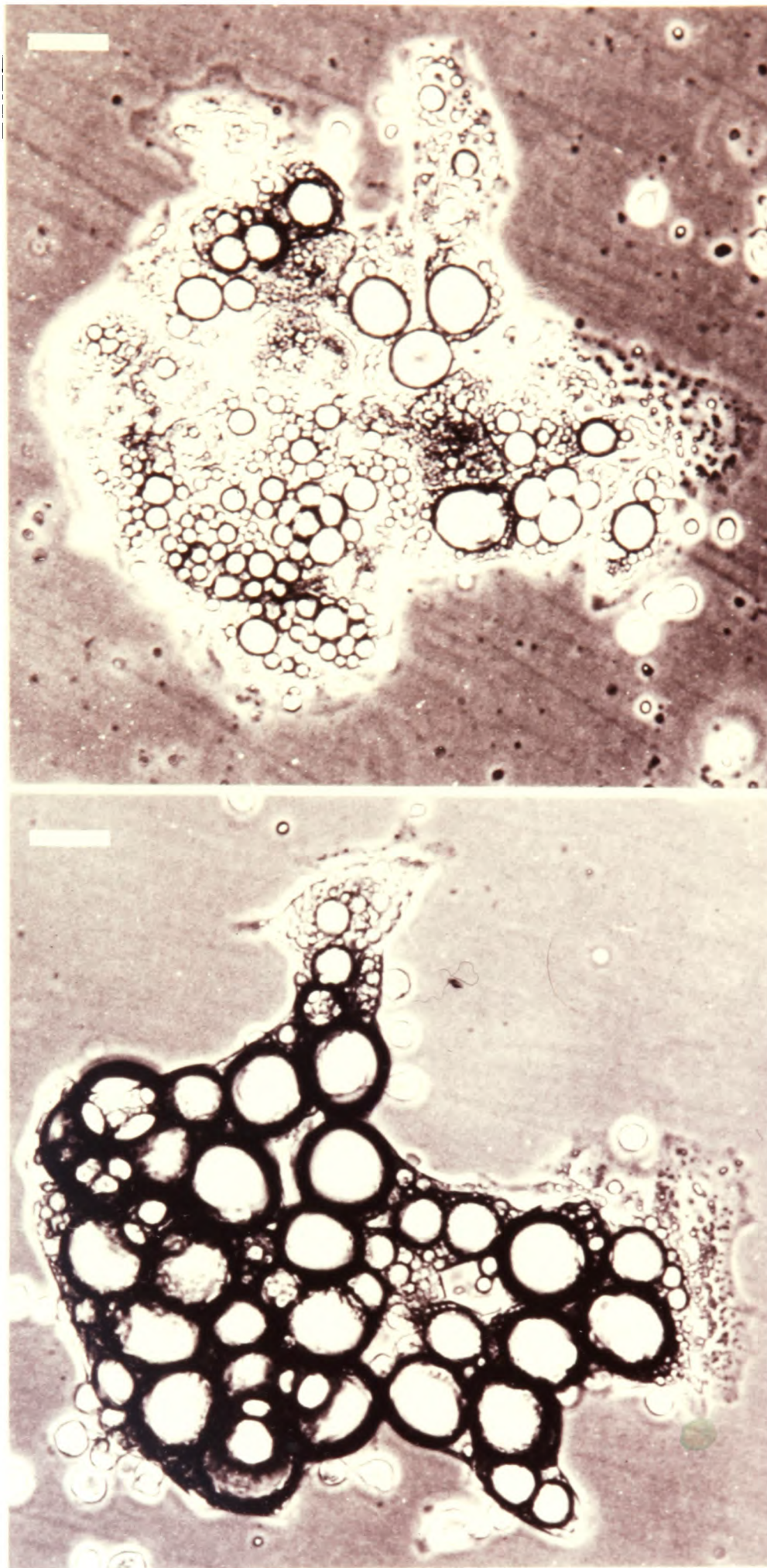


Plate 4.1.

Phase contrast photomicrograph (A) showing a small, well differentiated, adipocytic colony, type I, 0.3 mm diameter, after 7 days in culture. Cells have a high content of lipid.

B is the same colony as in A, 0.3 mm diameter after 11 days in culture. The size of lipid vacuoles has increased and the colony has retained its type I morphology. The culture medium is BGJ_b with RbP unchanged. Bar is 40μm.

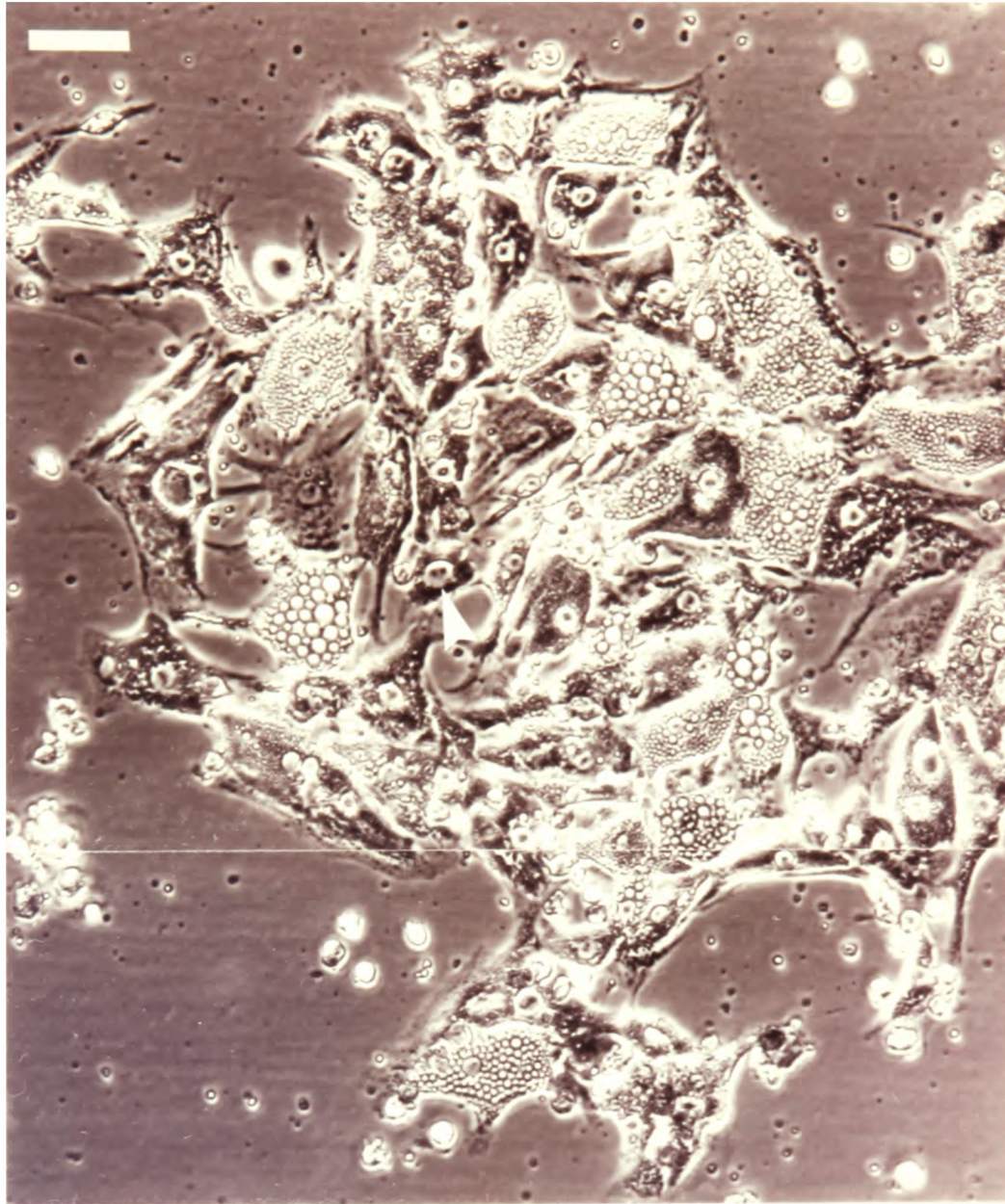


Plate 4.2.

Phase contrast photomicrograph to show a typical type II colony, cultured in BGJb with RbP, unchanged, on day 11 of culture. A large proportion of cells have a high lipid content yet cells characterized by dark, perinuclear granules can be seen (arrow). This colony has a much greater cell number than the type I colony in Plate 4.1 but fewer cells than the type III colony illustrated in Plate 4.3 Bar is 100 μ m.

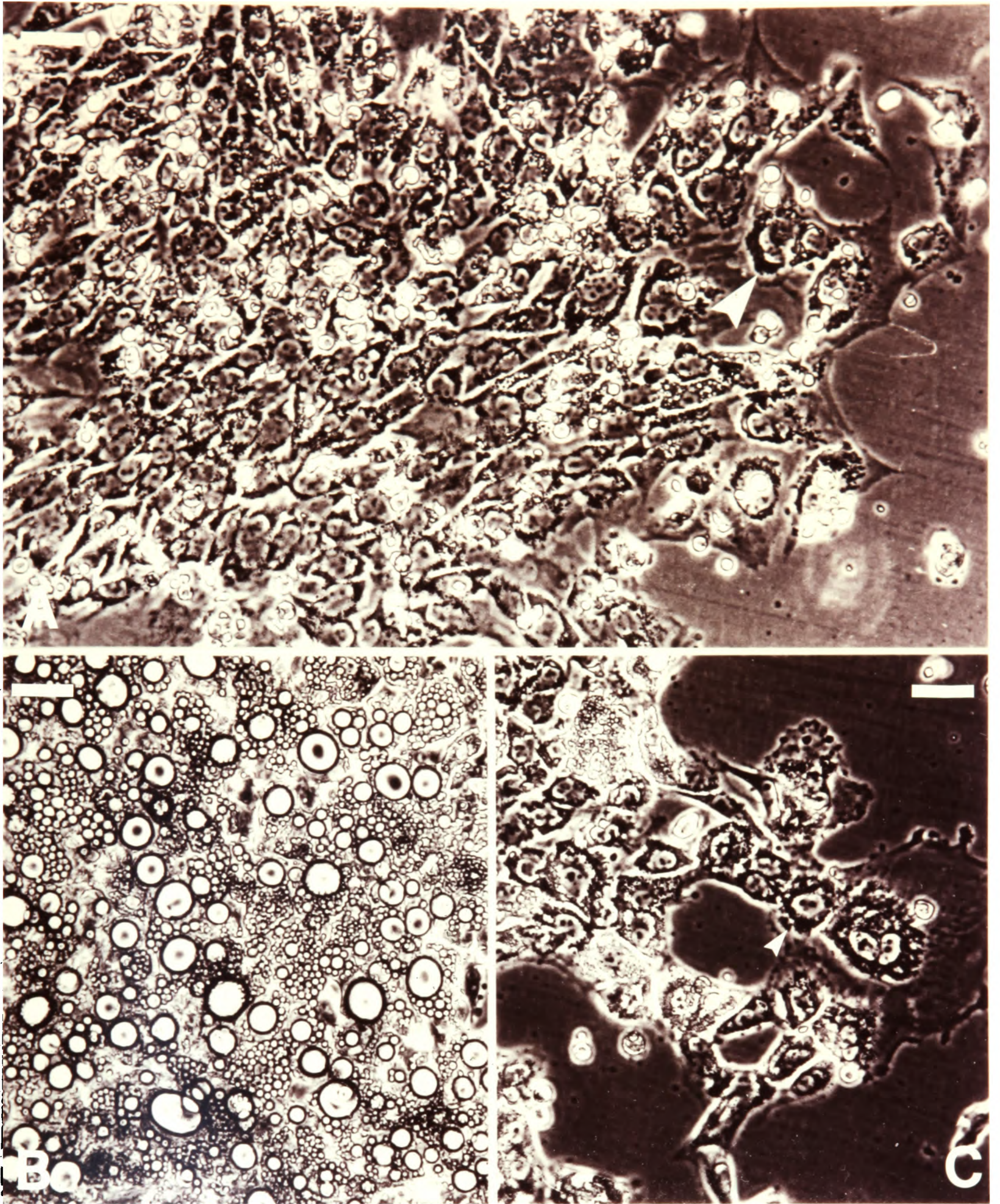


Plate 4.3.

Phase contrast photomicrographs to show the major portion of a large, adipocytic, type III colony, 0.9 mm diameter, after 7 days in culture (A) characterized by small, polygonal cells with dark, perinuclear granules (arrow). Small lipid vacuoles may be seen in some cells.

B and C show the center and edge of the same colony, now 1.7 mm diameter, after 11 days in culture. The culture medium is BGJ₆ with RbP, unchanged. B shows highly differentiated adipocytes with large lipid vacuoles. C shows peripheral cells. Some are well spread, others have a small, polygonal morphology. All have a high content of dark perinuclear granules, (arrow). Bar is 40 μ m.

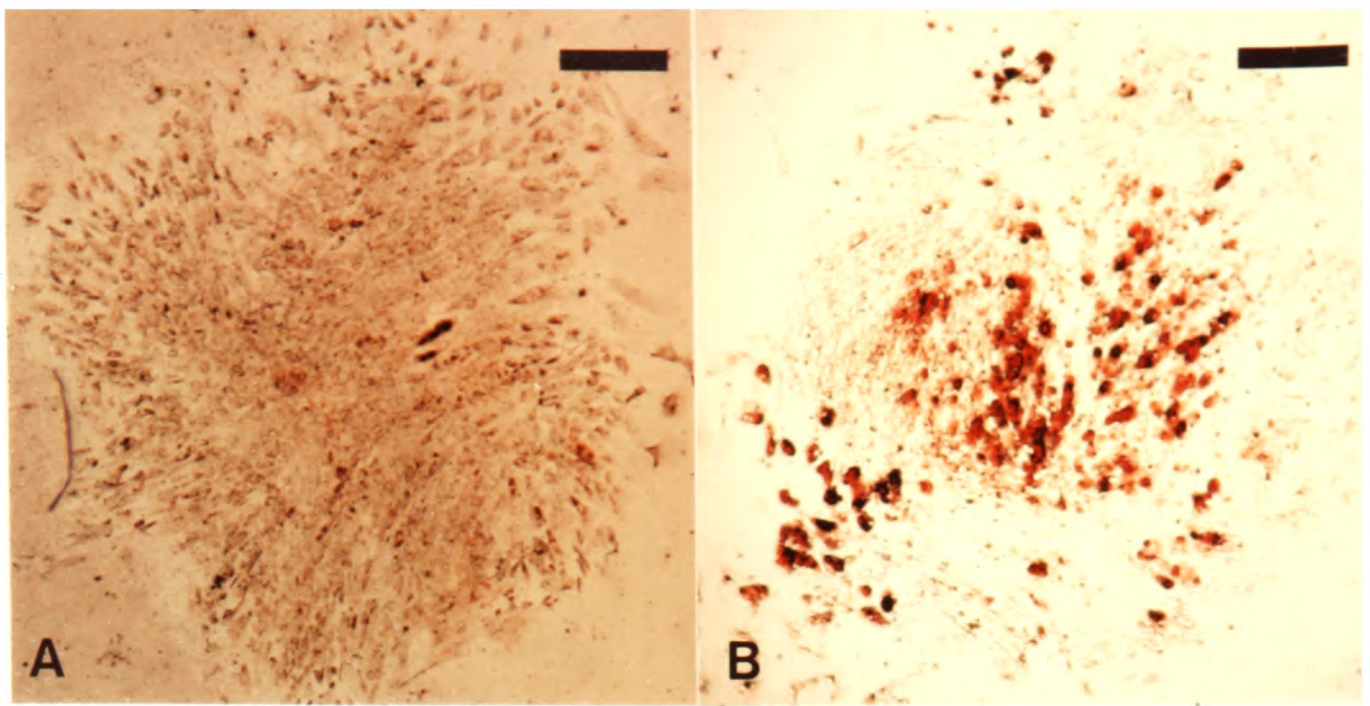
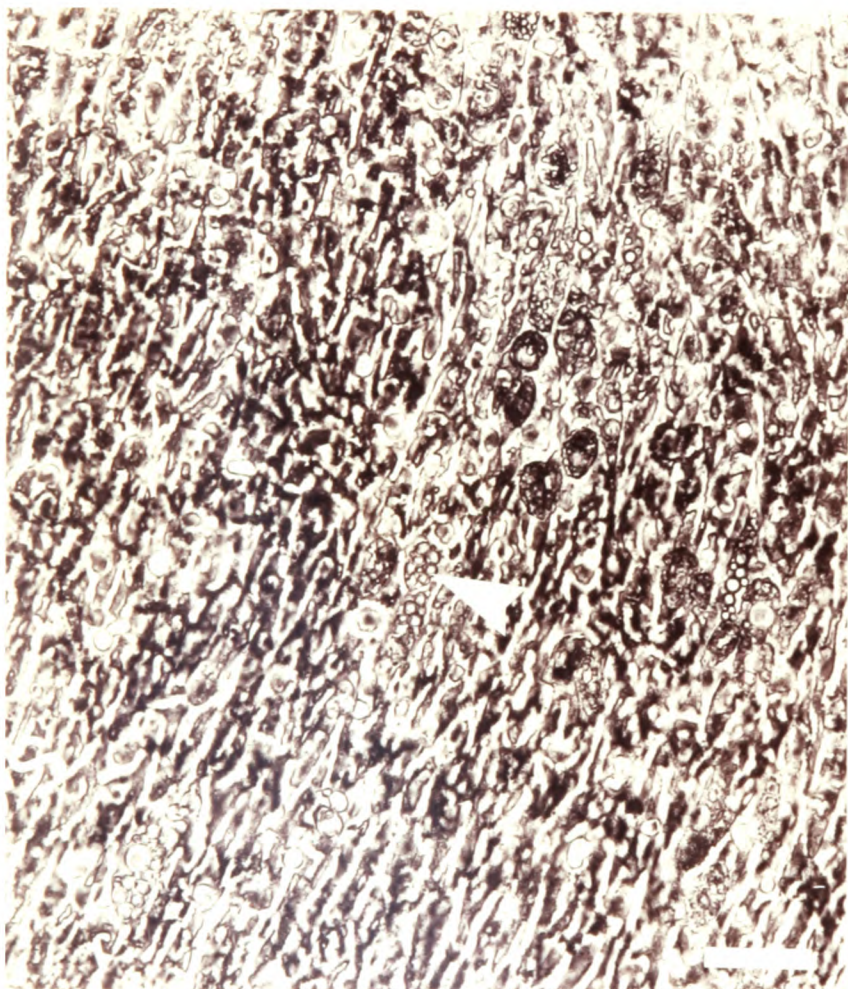


Plate 4.4 A and B.

Photomicrographs to show two colonies (A and B) cultured in BGJb with 15% RbP, fixed in formol calcium and stained with Oil-Red-O to localize lipid. A and B differ widely in the amount of fat expressed. Bar is 350 μ m.



C

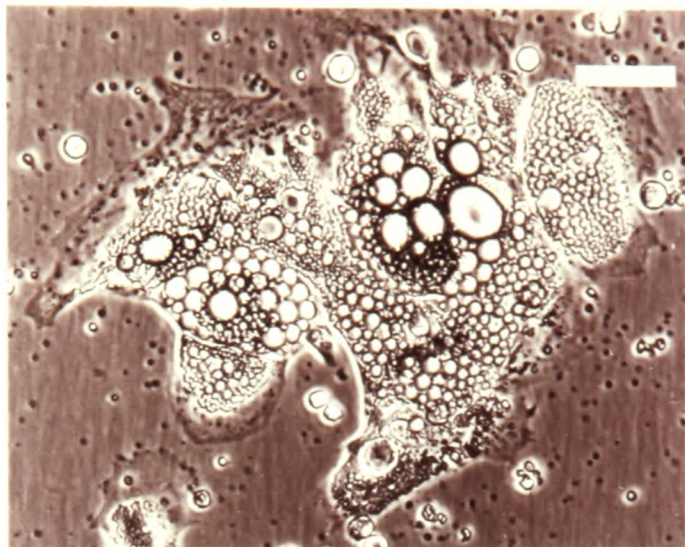


D

Plate 4.4 C and D.

C and D are phase contrast photomicrographs of comparable adipocytic colonies, cultured in BGJb with RbP and Hc, to day 11. C was adipogenic at the ++ level. Fat cells can be seen (arrow) but were either solitary or in clumps of two or three. D was adipogenic at the +++ level. Fat cells are apposed to each other in groups, separated by granular cells. Contrast this with the colony in Plate 4.3, which, on day 11, was fatty at the ++++ level. In Plate 4.3b the center of the colony appears wholly adipogenic. Bar is 100 μ m.

A



B

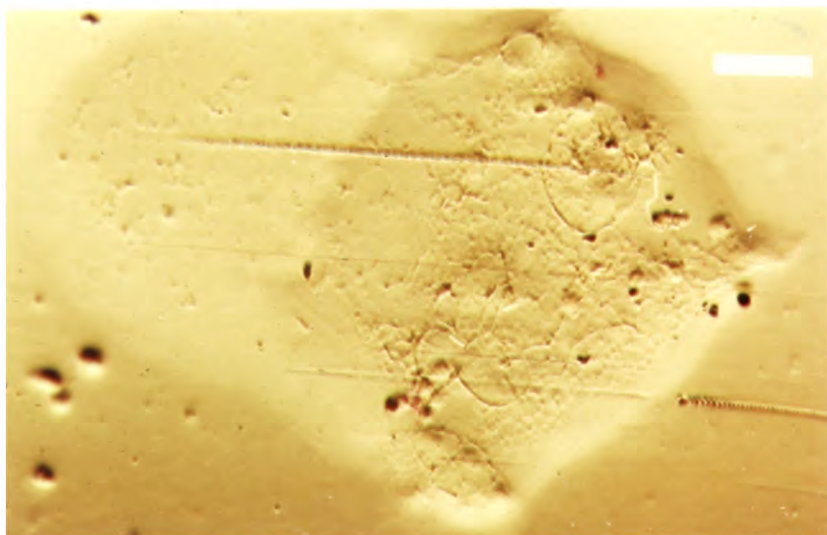


Plate 4.5.

A is a phase contrast photomicrograph of a type I colony on day 11 of culture. Bar is 65 μ m.

B is a photomicrograph of the same colony following incubation in ^3H -Thymidine, fixation in 95% alcohol, histochemical localization of AP activity and autoradiography. Bar is 50 μ m.

There is no evidence of AP activity or ^3H -Thymidine uptake in this colony.

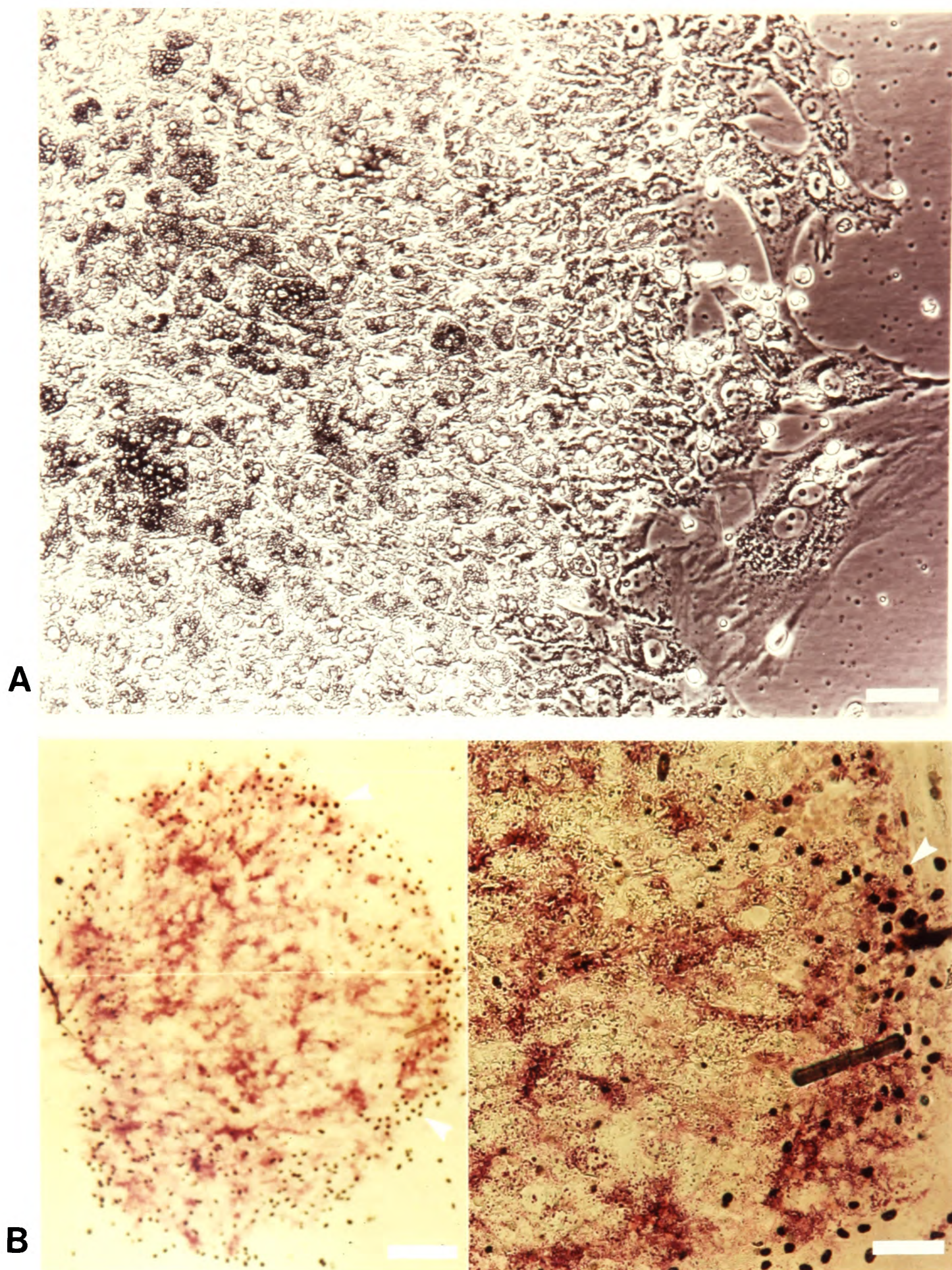


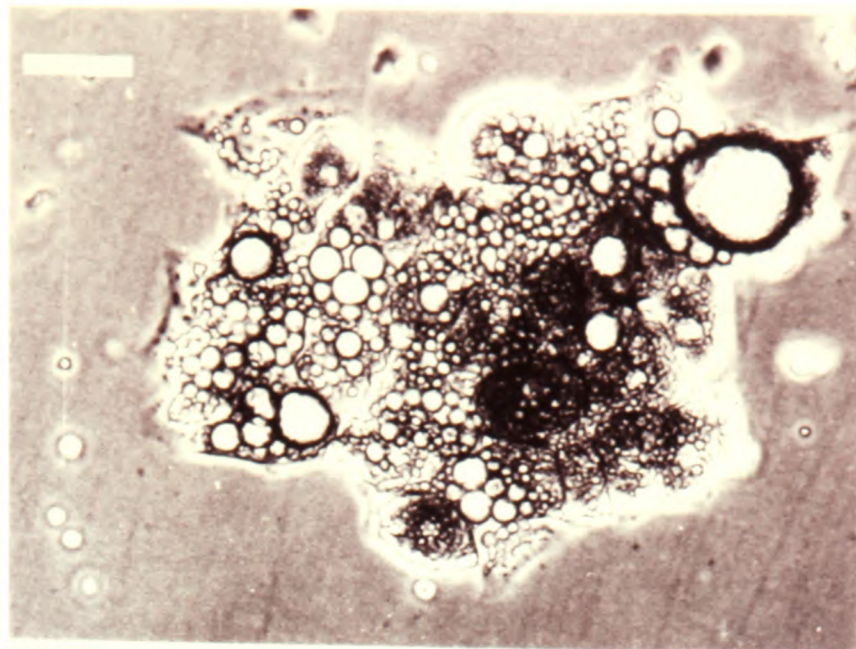
Plate 4.6.

A is a phase contrast photomicrograph of a type III adipocytic colony on day 11 of culture. Bar is $65\mu\text{m}$.

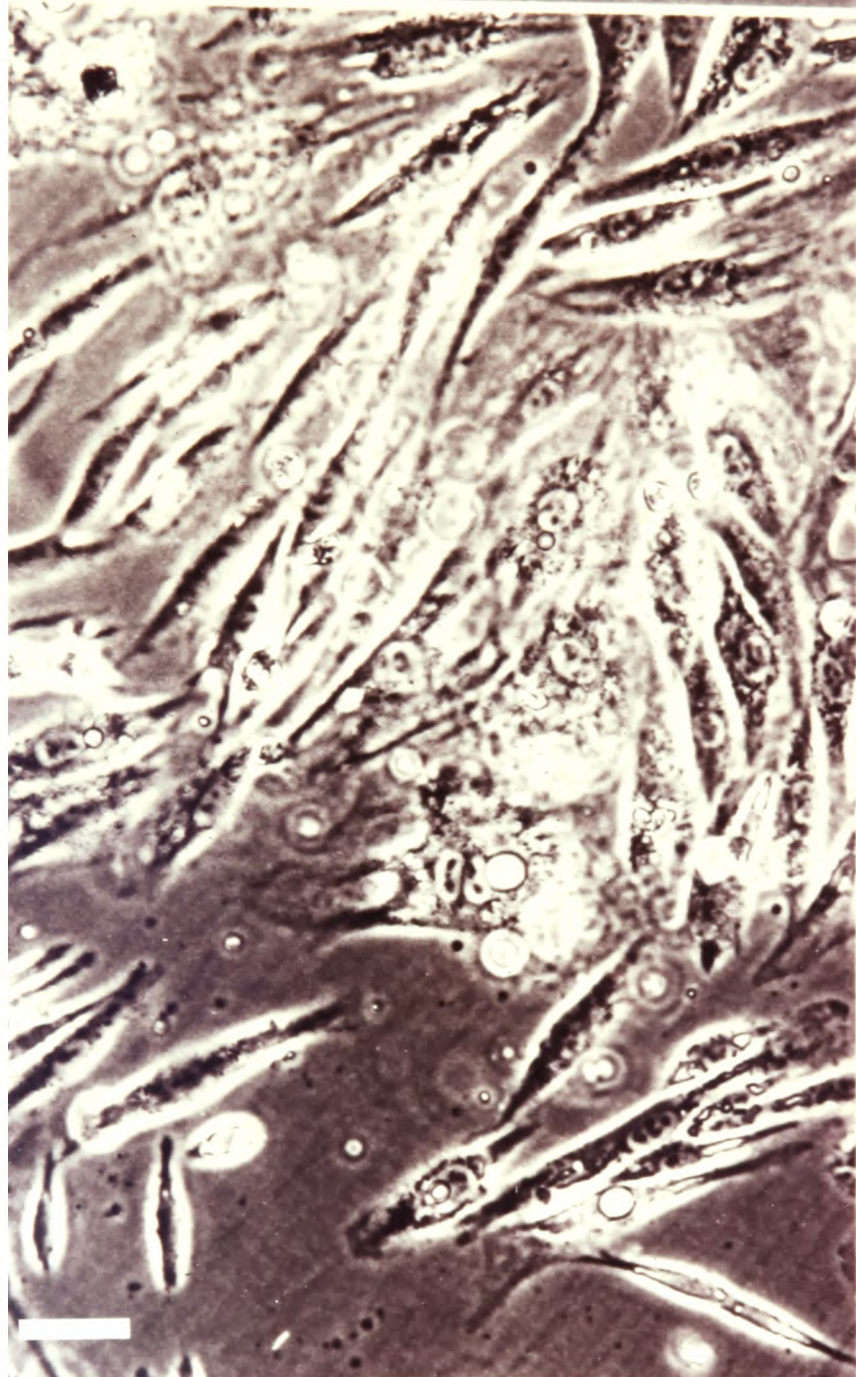
B is a photomicrograph of the same colony following incubation in ^3H -Thymidine, fixation in 95% alcohol, histochemical localization of AP activity and autoradiography. Bar is $270\mu\text{m}$.

C is a high power photomicrograph of B. Bar is $90\mu\text{m}$.

This series of photomicrographs demonstrates that adipocytic colonies can express AP activity, and that cells which take up ^3H -Thymidine are located towards the periphery (arrowed) with adipocytic cells centrally.



A



B

Plate 4.7.

A is a photomicrograph to show a small, adipocytic, type I colony, 0.25 mm diameter on day 7 of culture. Cells are characterized by a high lipid content. The culture medium was BGJ_b with RbP changed to BGJ_b with FCS on day 8.

B shows the major portion of the same colony as in A on day 11 of culture. The diameter is now 0.7 mm. Most of lipid has gone and cells have typical fibroblastic appearance. Bar is 40 μ m.

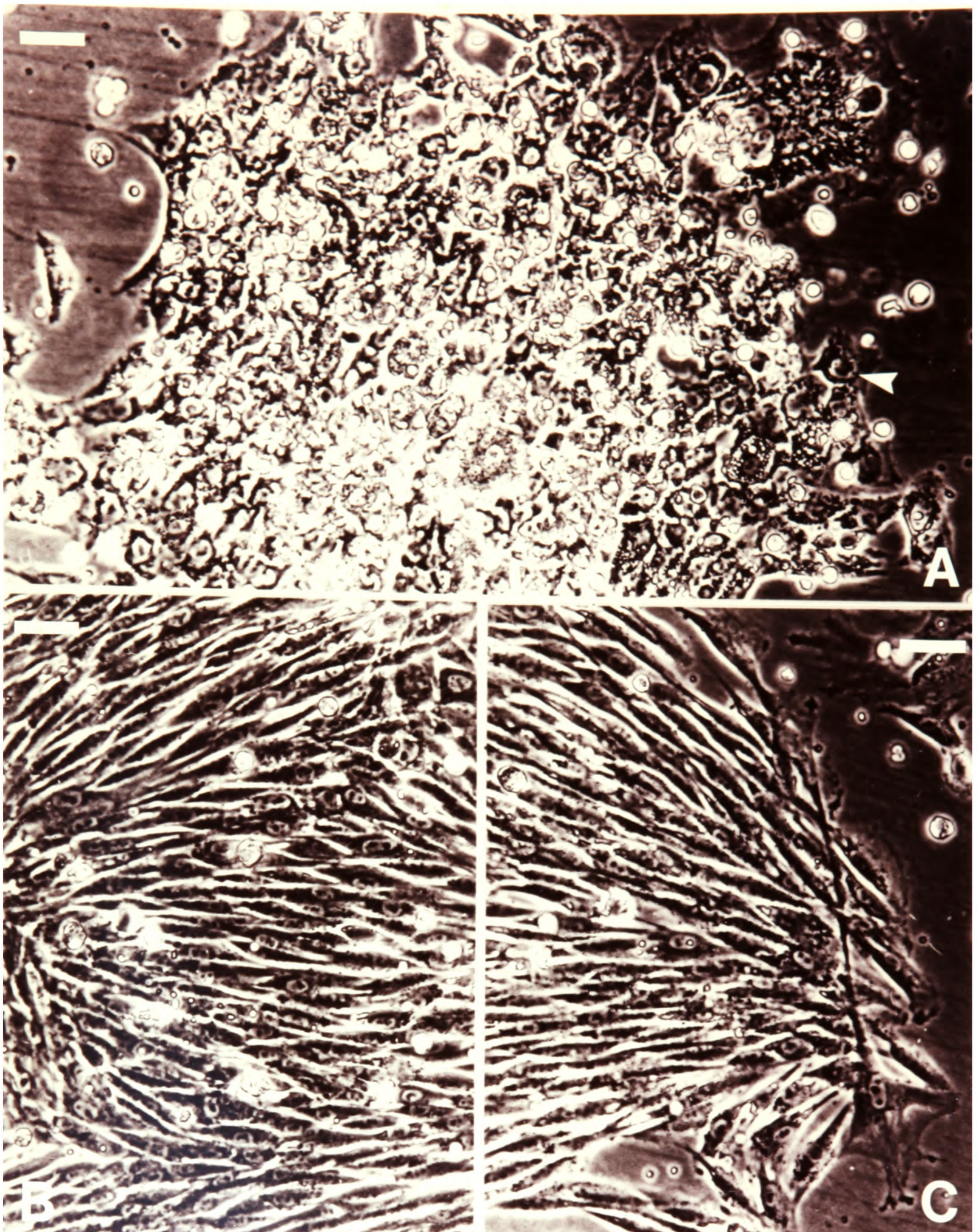


Plate 4.8.

Phase contrast photomicrograph to show the major portion of large, adipocytic, type III colony, 0.8 mm diameter, after 7 days in culture. Tightly apposed polygonal cells with dark, perinuclear granules (arrow), and small lipid vacuoles, similar to those illustrated in Plate 4.3 can be seen (A). The culture medium was BGJ_b with RbP changed to BGJ_b with FCS on day 8.

B and C show the center and edge, of the same colony, now 3.1 mm diameter on day 11. The lipid and perinuclear granules have largely disappeared and the cells in the center (B) and edge of colony (C) have a typical fibroblastic morphology. Bar is 40 μ m.

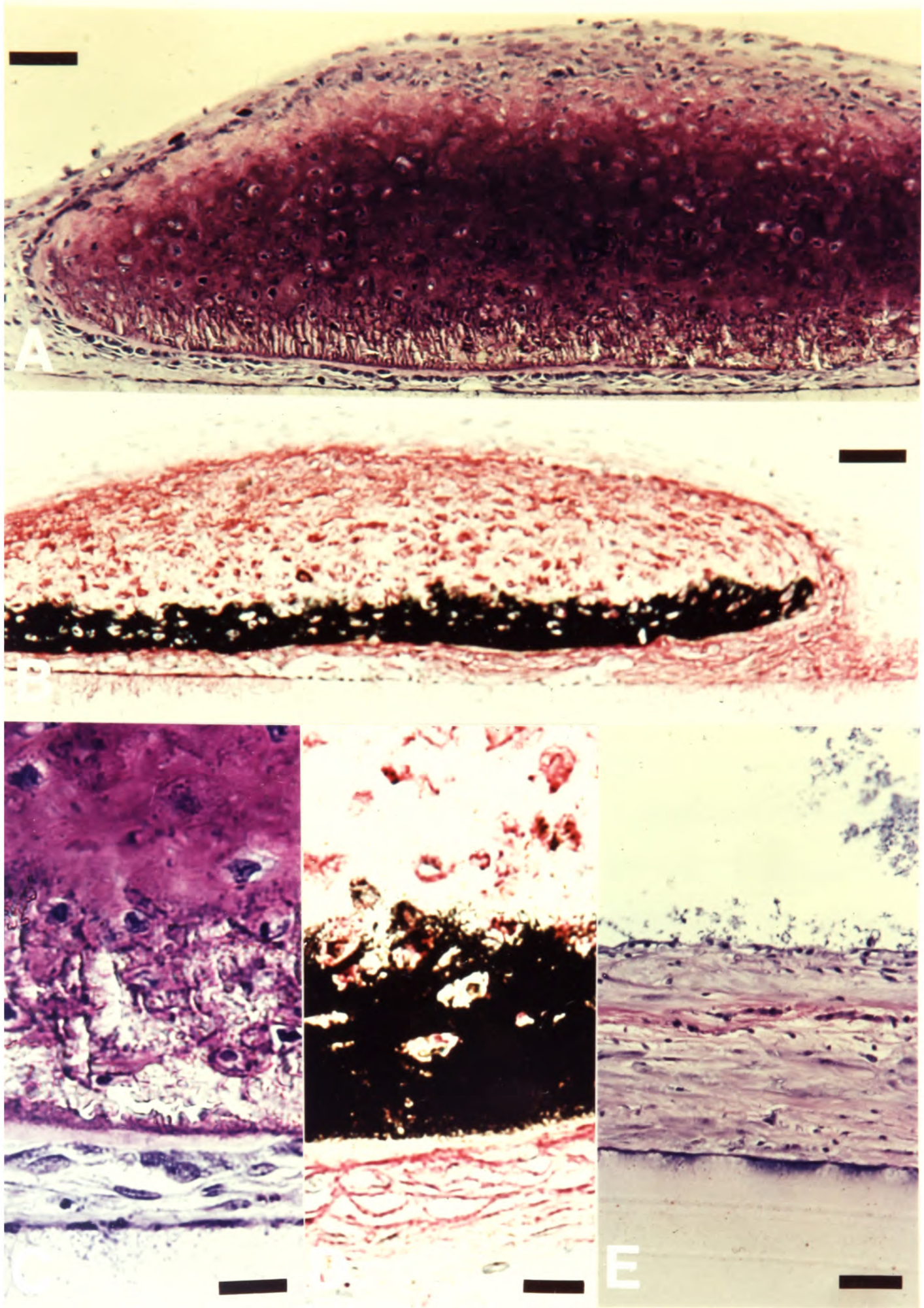


Plate 4.9.

Photomicrographs of tissue in a DC formed by a cell population expanded from colony 67, an adipocytic colony.

A is stained with toluidine blue and B is stained to localize AP activity and counterstained with Von Kossa.

Bar is 65 μ m.

C and D are high power views of A and B respectively. E shows a chamber containing fibrous tissue lying adjacent to the millipore filter. Bar is 14 μ m.

CHAPTER 5. MARKERS FOR THE MARROW ADIPOCYTE PHENOTYPE.

5.1: INTRODUCTION.

In view of their shared origin with the osteoblast, and their role in providing an appropriate environment for the differentiation of cells of the granulocyte-monocyte lineage, marrow adipocytes are of considerable interest. Despite their biochemical and morphological similarity to extra medullary adipocytes there are distinct differences (chapter 1.9).

Extra medullary adipocytes, for example have been studied extensively in vitro and can be induced to differentiate in response to insulin. Medullary adipocytes in culture, however, remain unaffected by insulin, and differentiation is promoted by hydrocortisone or dexamethasone (Greenburger, 1978; 1979).

Differentiation in extra-medullary adipocytes has been studied extensively at a morphological and biochemical level. Like medullary adipocytes and osteoblasts the extra-medullary adipocyte is derived from an undifferentiated mesenchymal stromal-vascular precursor cell (Ailhaud, 1982; Ailhaud et al, 1987). Using cell populations derived from fatty tissues and cloned pre-adipocyte cell lines (chapter 1.9), phenotype specific and stage specific differentiation markers have been characterised both at the protein and mRNA levels, making the extra-medullary adipocyte the best characterised of any cell type derived from a stromal-vascular precursor.

Expression of adipocyte differentiation markers in marrow adipocytes has been largely unstudied although expression of the adipocyte differentiation markers adipsin and PAL422(AP2) was recently reported in the murine marrow derived pre-adipocyte cell line BM20 (Gimble et al, 1989; 1990). Because of the similarities between the extra-medullary and medullary adipocytes it is of great interest to determine whether these differentiation specific markers are expressed in marrow adipocytes and if so, whether they may be used as differentiation markers in the study of marrow stromal lineages.

PAL422(AP2) is 14KDa intra-cellular fatty acid binding protein coded by a 0.7Kb cDNA, extracted from the murine pre-adipocyte 3T3-L1 cell line (Lane et al, 1990; Bernlohr et al, 1984), and is widely regarded as a late stage specific adipocyte differentiation marker. In this chapter, expression of PAL422(AP2) was investigated in marrow adipocytes at the protein and mRNA levels in tissue derived from the rat and the rabbit.

5.2: RESULTS.

5.2a: MORPHOLOGICAL OBSERVATIONS ON RAT MARROW STROMAL CULTURES.

When single cell suspensions derived from pooled femoral midshaft marrow were cultured in medium supplemented with 20% FCS, an attached stromal layer with a characteristic fibroblastic morphology was formed (Plate 5.1a). Adipocytic differentiation was rare in cultures with FCS only. When cells were cultured in 20% FCS with 10^{-7} M Hc, or initiated in 20% FCS and then changed to medium with 20% FCS and Hc, a proportion became rounded and acquired a perinuclear ring of granules which, with time, fused to form refractile lipid vacuoles in the cytoplasm, typical of an adipocytic morphology (Plate 5.1d). When cultures were established in medium supplemented with 15% rat plasma (RtP) the amount of adipogenesis was much greater and the majority of the cells in the cultures became adipocytic. In these cultures pre-adipocytes developed first and were characterized by a typical polygonal, or rounded morphology with a dense granular cytoplasm. Subsequently, cells packed with cytoplasmic lipid vacuoles developed and were seen distributed widely throughout the cultures. Plate 5.1b shows a RtP culture on day 9, illustrating cells characterized by a dense granular cytoplasm. Plate 5.1c shows the same culture on day 14. Lipid laden cells are now widespread throughout the culture. The morphology of the cells was similar to that described previously for rabbit cells

grown in medium with autologous plasma (Plates 4.1, 4.2 and 4.3).

5.2b: EXPRESSION OF THE ADIPOCYTE SPECIFIC PROTEIN
 PAL422(AP2) IN RAT TISSUES BY WESTERN BLOTTING.

Rat Marrow Stromal Cultures.

Cultures initiated and maintained in medium with 20% FCS were almost completely without adipocytes and were compared with cultures set up in conditions known to promote adipogenesis. These included marrow cells cultured in medium with 20% FCS changed to that with 20% FCS and 10^{-7} M Hc on day 7, and marrow cell cultures established in medium supplemented with FCS changed to that with 15% rat serum on day 7. Extracts from fresh rat femoral marrow and from differentiating cultures of the murine pre-adipocytic cell line 3T3-L1 (chapter 2.4b), a known positive control, were also tested. After 17 days, extracts were prepared from the cultures and proteins extracted (chapter 2.6a).

On average, two semi-confluent 25cm² tissue culture flasks gave 200-300 μ l of extract with 0.6-1.2 μ g protein per μ l. When 30 μ g samples of each extract were compared for expression of the adipocyte specific PAL422(AP2) protein by western blotting, a 14KD protein was identified in lanes containing extracts from marrow cells cultured with Hc (Plate 5.2 lane (iii)) and with rat serum (Plate 5.2 lane (iv)). The protein was also

identified in extracts from fresh femoral rat marrow (Plate 5.2 lane (i)) and of 3T3-L1 cells used as a positive control (Plate 5.2 lane (v))

In another blot, (Plate 5.3), an extract from rat peri-abdominal fat is compared with extracts of marrow cells cultured from day 0 in BGJb medium and 20% FCS or 15% RtP rather than rat serum. 3T3-L1 pre-adipocytic cell extracts were used as a positive control. The extra-medullary fat extract showed a positive reaction for the PAP422(AP2) protein (Plate 5.3 lane (i)) as well as the extracts of marrow stromal cells cultured in RtP and 3T3-L1 (Plate 5.3 lanes (ii) and (iv)). Thus, by comparing Plate 5.3 lane (ii) and Plate 5.2 lane (iv), it can be seen that both RtP and rat serum support PAL422(AP2) expression.

A faint positive band was seen in the extract from marrow cells cultured in medium with FCS only (Plate 5.3 lane (iii)). Although there is very little expression of adipogenesis in rat marrow cells cultured with FCS only, this can vary from one batch of FCS to another. The results in Plate 5.3 were obtained with a different batch of FCS to those in Plate 5.2. However, the result in Plate 5.3 lane (iii) was only rarely seen. Commonly, extracts of marrow cells cultured in medium supplemented with 20% FCS did not show evidence of adipogenesis as illustrated in Plate 5.2 lane (ii).

Rat Osteosarcoma Derived Cell Lines.

In contrast with the primary marrow stromal cultures, cells from the rat osteosarcoma cell lines ROS 17/2.8 and UMR 106 did not show signs of adipogenesis in vitro or express PAL422(AP2) when extracts of the cells cultured in 20% FCS with 10^{-7} M Hc were tested by western blotting using the PAL422(AP2) antiserum. The results for western blots of all tissues investigated are summarized in Fig 5.1.

5.2c: NEGATIVE CONTROLS.

As a negative control the nitrocellulose membrane was, following blotting, divided into two. One half was tested with antiserum and the other with normal rabbit serum at the same dilution. Plate 5.4b, the control blot for Plate 5.4a is shown as an example of a negative control. Negative controls for other blots are not shown.

5.2d: EXPRESSION OF THE ADIPOCYTE SPECIFIC PROTEIN PAL422(AP2) IN RABBIT TISSUES BY WESTERN BLOTTING.

When protein extracts prepared from extra medullary fat and yellow marrow were compared with 3T3-L1 cell extracts by protein blotting using the anti PAL422(AP2) antiserum, a 14KD protein band was seen in the 3T3-L1 extract (Plate 5.4a -lane (i)) and extra medullary fat extracts (Plate 5.4a - lanes (iii) and (iv)), but not in extracts from rabbit yellow marrow

(Plate 5.4a - lane (ii)). When primary marrow stromal cultures were grown under conditions which promoted either a fibroblastic or an adipocytic morphology i.e, with FCS or with RbP as a serum supplement (chapters 3 and 4) there was no evidence of PAL422(AP2) expression on western blotting (Fig 5.1).

SPECIES	TISSUE TESTED	RESULT
MOUSE	3T3-L1 Adipocytic Culture	+++
RABBIT	Extra-Medullary Fat Extract Marrow Extract 1° Marrow Adipocyte Culture 1° Marrow Fibroblast Culture	++ - - -
RAT	Extra-Medullary Fat Extract Femoral Marrow Extract 1° Marrow Adipocyte Culture 1° Marrow Fibroblast Culture Cultured ROS 17/2.8 Cells Cultured UMR 106 Cells	+++ ++ ++ - - -

Fig 5.1 Results of Western Blots to compare PAL422(AP2) expression in a range of tissue extracts. Positive responses are graded + to +++ subjectively indicating the intensity of the colour reaction.

5.2e: EXPRESSION OF PAL422(AP2) AND COLLAGEN TYPE I mRNA
IN CULTURES OF RAT MARROW STROMAL CELLS.

Cells were grown to confluence, replated at 5×10^3 per cm_2 and allowed to become confluent in 1st passage culture before the RNA was prepared. About 2×10^7 cells, approximately the number found in two confluent 75cm^2 tissue culture flasks, gave a

minimum quantity of mRNA for subsequent analysis. Details of the cell culture and mRNA extraction techniques are given in chapter 2.7. Three different conditions were used.

Conditions CC; primary cultures, initiated in medium with 20% FCS (condition C) were passaged into the same (condition C).

Conditions CD; primary cultures, initiated in medium with 20% FCS (condition C) were passaged into that supplemented with 20% FCS and 10^{-8} M Dexamethasone (DEX) (condition D).

Conditions DD; primary cultures, initiated in medium with 20% FCS and DEX (condition D) were passaged into the same (condition D).

Northern blots were prepared and probed using cDNA probes to PAL422(AP2) or Collagen type I mRNA. Under each condition probing for collagen type I message revealed two bands representing the two transcripts of the collagen pro $\alpha 1(1)$ chain at 4.7Kb and 5.7Kb (Plate 5.5) (Genovese et al, 1984). Under conditions CD, however, rather less pro $\alpha 1(1)$ collagen mRNA was seen than in the other two conditions. With PAL422(AP2) cDNA, a 0.7Kb band representing AP2 mRNA was seen in RNA from cells cultured under conditions CD whereas a smaller amount of PAL422(AP2) mRNA was seen in that from

conditions DD. No evidence of AP2 mRNA was found in RNA from conditions CC.

5.2f: LOCALIZATION OF PAL422(AP2) PROTEIN IN FIXED CULTURES BY IMMUNOCYTOCHEMISTRY.

In order to correlate the results of PAL422(AP2) mRNA expression with in situ protein expression cultures were set up using the three conditions CC, CD and DD, fixed and stained using the rabbit anti mouse antiserum to PAL422(AP2). Parallel cultures were stained with normal rabbit serum at an equivalent dilution as a negative control.

In semi-confluent CD and DD cultures discrete areas of adipogenesis were observed, separated by cells which retained a fibroblastic appearance. Adipocytic cells were rare under conditions CC. In general, staining was localized to adipocytic areas (Plate 5.6 (ii) and (iii)), whilst there was little staining in the intervening non adipogenic area in between. Within the adipocytic areas protein was mostly but not exclusively localized to cells with cytoplasmic lipid vacuoles, however, sometimes cells with fat vacuoles or no fat vacuoles at all expressed the protein (Plate 5.7 (ii) and (iii)). Conversely, well developed adipocytes were sometimes seen which did not express the protein particularly under conditions DD (Plate 5.7 (iii)). Occasional cells were sometimes seen expressing PAL422(AP2) under conditions CC (Plate 5.6 (i) and Plate 5.7 (i)).

5.3: DISCUSSION.

Adherent marrow stromal populations have osteogenic potential, and evidence has been presented to show that adipocytic marrow stromal colonies have osteogenic potential and form bone in DC's. An objective was to establish if markers of adipocytic differentiation in extra-medullary fat or pre-adipocytic cell lines were expressed in primary marrow stromal cultures in vitro. The results demonstrate that PAL422(AP2) is expressed at both the protein and mRNA levels, and that its expression could be localized immunohistochemically to adipocytes. PAL422(AP2) is thus of value as a stromal marker in exploring lineage relationships between populations of marrow stromal cells. This is believed to be the first report of PAL422(AP2) expression in a primary culture of marrow stromal cells and in a population with known osteogenic potential.

Expression of PAL422(AP2) was not seen in rabbit adipogenic marrow cultures. This was not surprising since the antiserum used was raised in rabbits. On the other hand rabbit extra-medullary fat extract gave a positive band at about 14Kd, the M_r of PAL422(AP2) on western blotting, at an identical M_r to the band from the 3T3-L1 extract, used as a positive control. Absence of PAL422(AP2) in adipogenic rabbit marrow cultures could, therefore, be interpreted as a species difference from the rat.

An alternative explanation might be that, because the

antiserum was raised in rabbits, it was unable consistently to recognise epitopes on rabbit proteins. Conclusions about PAL422(AP2) expression in rabbit marrow adipocytes must, therefore, await the application of cDNA probes to mRNA prepared from rabbit marrow adipocytes, or the availability of an antiserum raised in a species other than the rabbit.

Either dexamethasone or hydrocortisone were essential for fat and PAL422(AP2) expression. Both have been reported to promote adipogenic conversion in marrow stromal cultures and pre-adipocytic cell lines (Greenburger, 1978; 1979; Lanotte et al, 1982; Kodama et al, 1982), observations which were confirmed in these experiments. Both glucocorticoids facilitated fat expression in the presence of a 20% FCS supplement. Unknown factors present in FCS, however, promoted adipogenic conversion in the absence of a glucocorticoid supplement, but the ability to support fatty differentiation varied widely from batch to batch of FCS. Dexamethasone promoted PAL422(AP2) mRNA expression in secondary culture but the amount of PAL422(AP2) message from cultures where the primary and secondary were supplemented with dexamethasone (conditions DD) was not as great as the amount detected when cells were cultured with a dexamethasone supplement in secondary alone (conditions CD).

Single marrow cell suspensions were explanted into primary culture at a density of 1.6×10^6 nucleated marrow cells cm^{-2} . These were mostly cells of the haemopoietic lineages with a

tiny number of stromal precursors that subsequently form the adherent stromal layer. Cultures were, therefore, effectively established in the presence of a haemopoietic feeder layer.

The importance of a haemopoietic feeder layer to the establishment of an adherent marrow stromal culture has been emphasised (Chapter 3), but the nature of the stimulus to stromal growth remains unknown. Growth factors produced by cells of the haemopoietic lineage, and thus of the non-adherent cell layer (Cannistra and Griffin, 1988; Sieff, 1987) could act directly or indirectly to promote stromal growth. It is also known that glucocorticoids can modulate the production of specific growth factors by haemopoietic cells. Hydrocortisone causes complex changes in myeloid populations in vitro, including an overall drop in the number of haemopoietic precursors (Metcalfe, 1969). Specific subsets of the myeloid population, however, appear to be selectively suppressed including eosinophil progenitors (CFU-Eo) (Slovic et al, 1985), lymphocytes (Gillis et al, 1979), granulocyte-monocyte precursors (GM-CFU) (McNeill and Fleming, 1973), erythrocytes and megakaryocytes. Hydrocortisone increased neutrophil progenitors (CFU-N) (Suda et al, 1983) and was essential for the formation of granulocyte colonies in serum free culture (Metcalfe, 1988). Hydrocortisone, therefore, has the potential to alter the composition of the non-adherent layer and the relative amounts of cytokines in the in vitro environment. Primary cultures in the presence of glucocorticoid are thus subject to a different

set of conditions to those in its absence. The basis of the effect on the adherent stromal layer remains unknown. One speculative explanation might be that primary culture in the absence of dexamethasone selected for a stromal population enriched for adipocyte precursors by modulating the synthesis of growth factors by the non adherent cells in the medium.

Collagen type I is the major collagen component produced by fibroblasts and osteoblast-like cells in vitro (Wiestner et al, 1981). Adipose conversion in 3T3-L1 cells has been characterised by down regulation of collagen type I mRNA with an increase in procollagen type IV transcription (Weiner et al, 1989), consistent with the formation of a basement membrane, a feature of the differentiated adipocyte phenotype. In this study, expression of PAL422(AP2) mRNA was associated with a marked reduction in collagen type I message, thus again, loss of collagen type I was a feature of adipocytic differentiation.

The experiments reported here were designed to integrate with those of Leboy et al (1991), carried out in the same laboratory, who used an identical protocol before probing with cDNA probes to collagen types I and III, osteonectin, AP and osteopontin. Their results are summarised in Fig 5.2.

cDNA Probe.	CC	CD	DD
Collagen I	1.3	0.3	1.0
Collagen III	1.6	0.4	1.0
Osteonectin	1.0	0.5	1.0
Alkaline Phosphatase	0.2	0.3	1.0
Osteopontin	0.0	0.3	1.0

Fig 5.2 Table of results adapted from Leboy et al, (1991) using comparable conditions and nomenclature to those described in the present study and probing with cDNA probes characteristic of the osteoblastic phenotype.

* Note that all values are obtained by densitometry of the autoradiographs and are expressed relative to the amount of hybridization seen using conditions 'DD'.

Cells exposed to dexamethasone in both primary and secondary culture (conditions 'DD') have higher levels of AP, osteonectin and osteopontin than those exposed to dexamethasone in secondary culture alone. Primary culture in the absence of dexamethasone, therefore, appears to favour adipocyte differentiation whilst in its presence, favours expression of the osteoblastic phenotype.

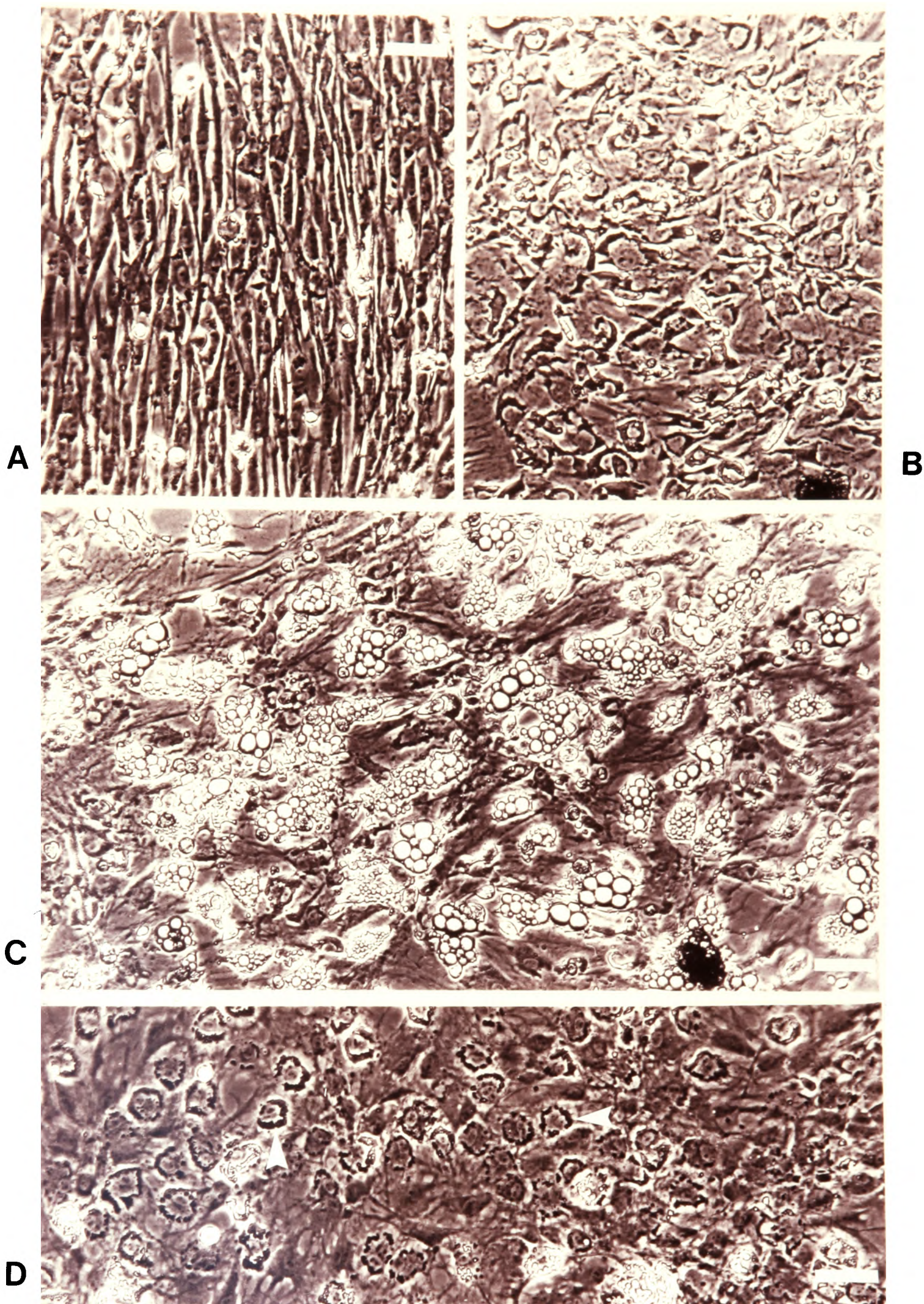


Plate 5.1.

Phase contrast photomicrograph of rat marrow stromal cells in live culture in BGJb supplemented with FCS on day 14 of culture (A). B shows cells in BGJb with RtP on day 9; and C is the same flask on day 14. The medium was unchanged. Cells in A are fibroblastic whilst in B, they have a more rounded appearance with a granular cytoplasm. In C, refractile lipid vacuoles were seen. D, culture medium BGJb, FCS and Hc, shows some cells with cytoplasmic lipid vacuoles. Others had a perinuclear granular morphology (arrowed). Bar is 60 μ m.

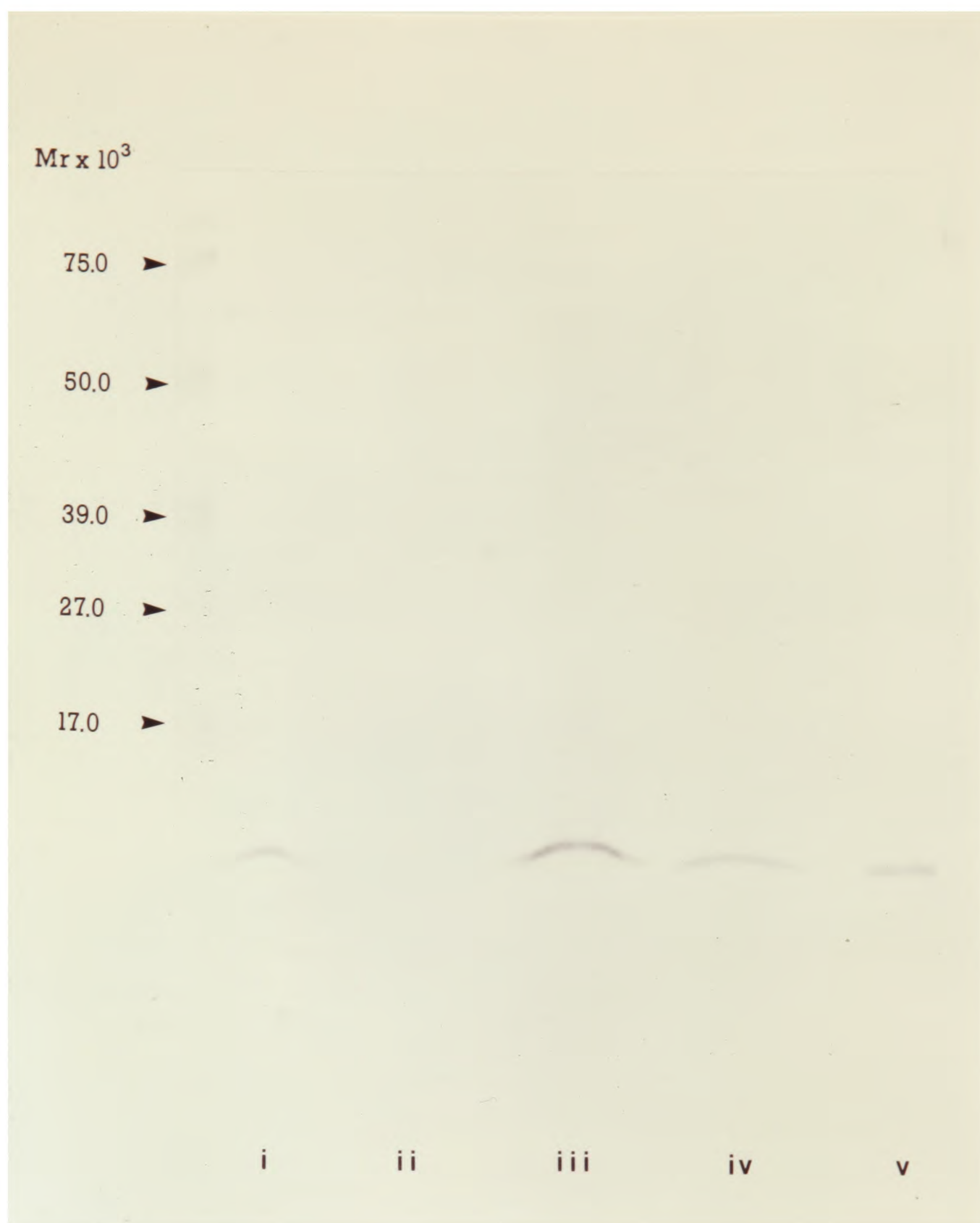


Plate 5.2

Western blot used to demonstrate immunoreactivity using PAL422(AP2) antiserum at a M_r of about 14K.

Lane (i) contains a femoral marrow extract; lane (ii), an extract of marrow stromal cells cultured in medium and FCS; lane (iii), a marrow stromal extract cultured in medium with FCS to which Hc had been added on day 7; lane (iv), a marrow stromal extract cultured with medium supplemented with rat serum and lane (v), a 3T3-L1 cell extract. Tissue was derived from rats.

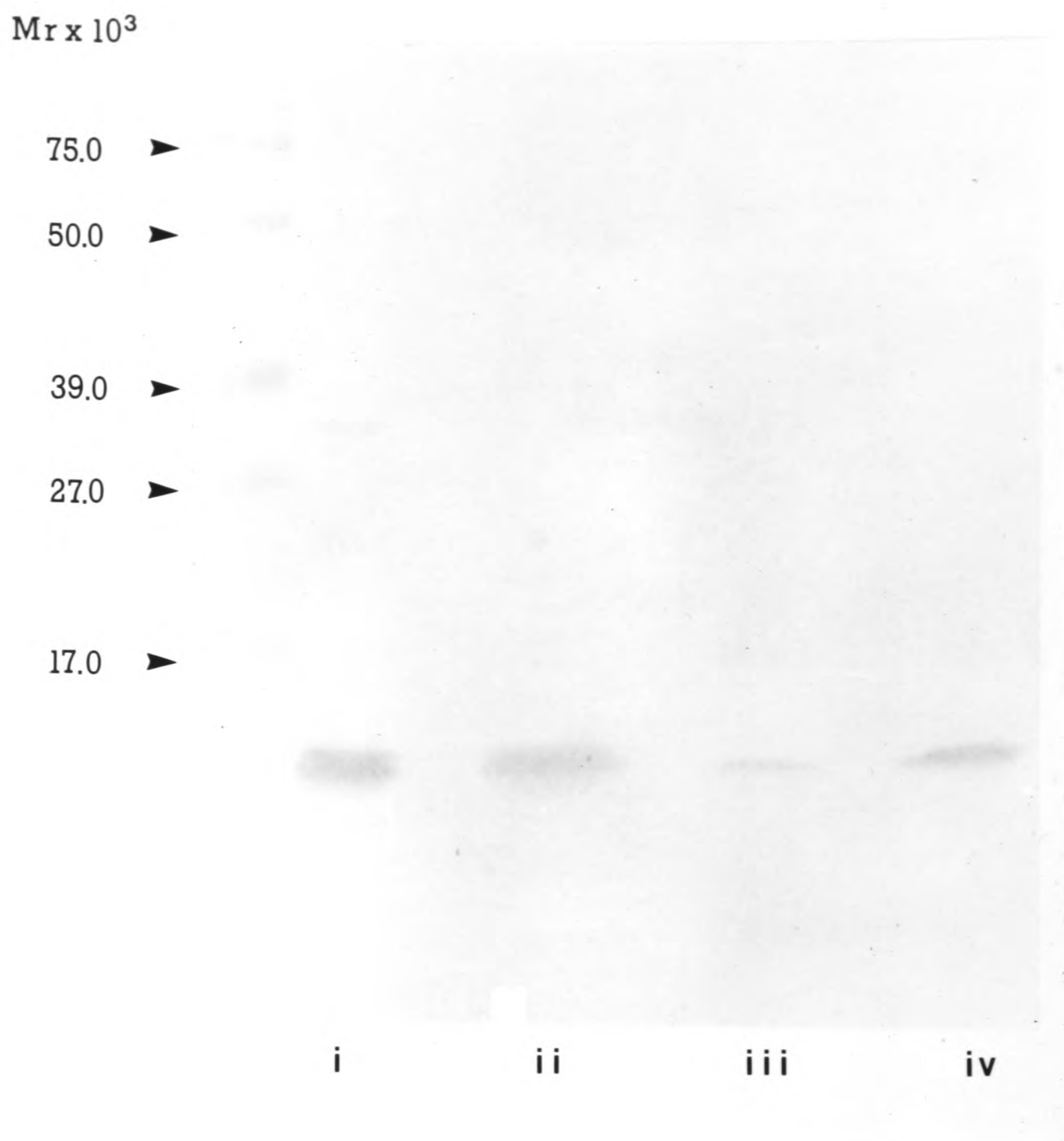


Plate 5.3

Western blot using AP2 antiserum to demonstrate immunoreactivity at a M_r of about 14K.

Lane (i) contains an extra-medullary fat extract; lane (ii) a marrow stromal cell extract cultured in medium supplemented with Rtp; lane (iii) an extract of cultured stromal cells in medium supplemented with FCS and lane (iv), a 3T3-L1 cell extract. Tissue was derived from rats.

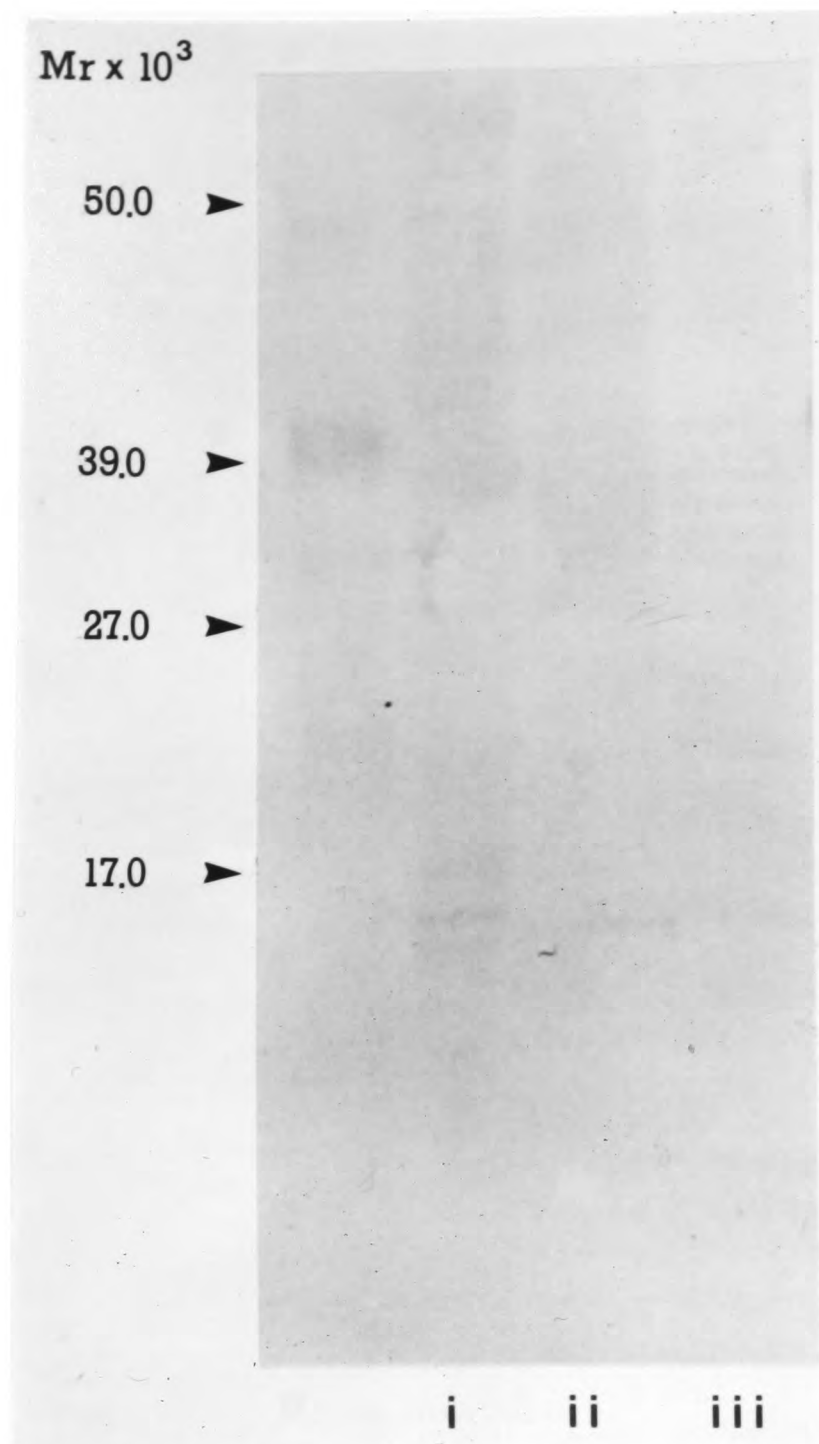


Plate 5.4b

Negative control for the blot shown in plate 5.4a. The blot was divided into two. The portion shown in plate 5.4a was treated with anti PAL422(AP2) antiserum whilst the portion shown here was treated with normal rabbit serum.

Lane (i), contains a 3T3-L1 cell extract; lane (ii), a yellow marrow extract and lane (iii), an extra-medullary fat extract from the same sample as used in plate 5.4a lane (iv).

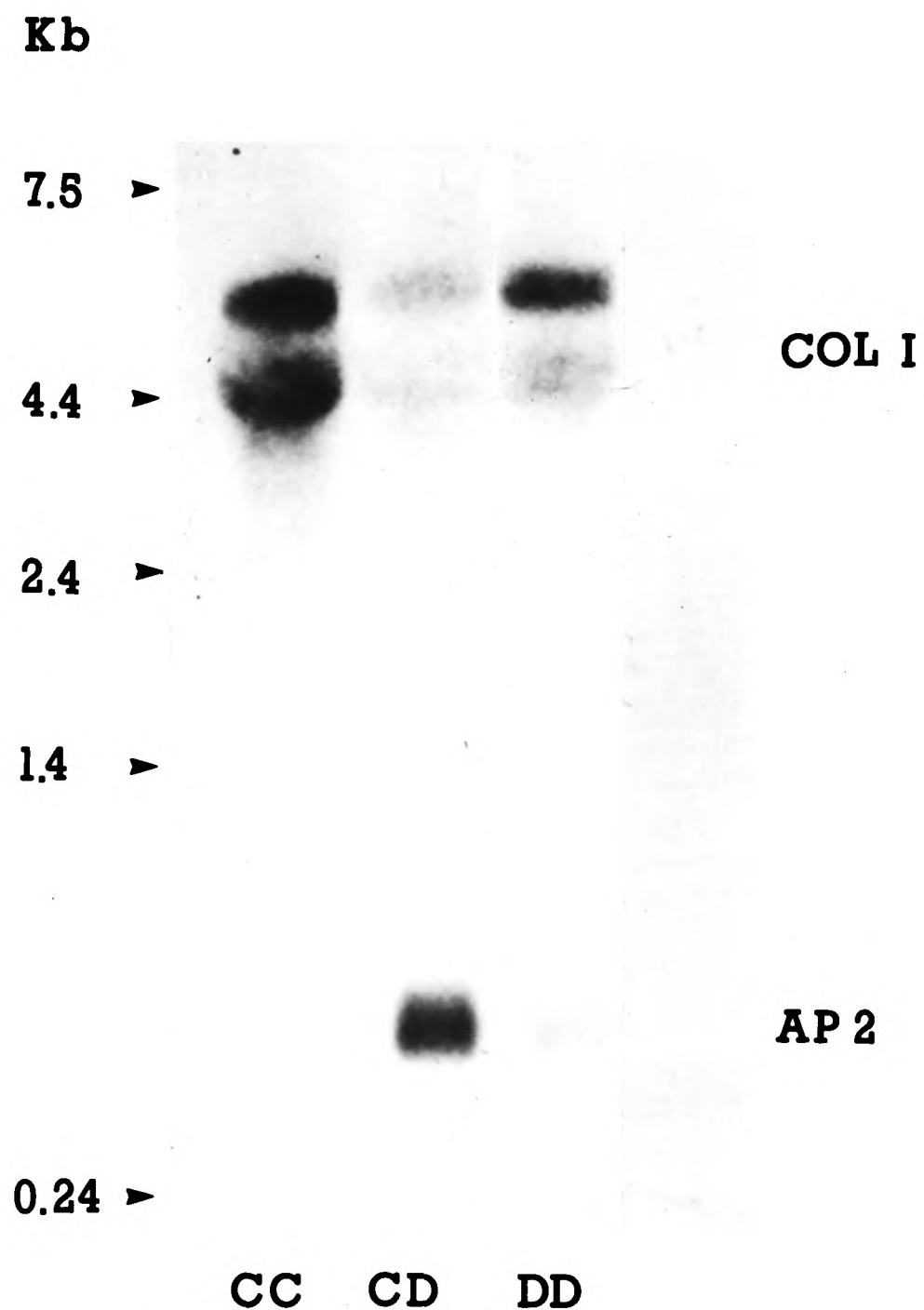


Plate 5.5

Northern blot of rat marrow stromal tissue cultured under conditions CC, CD and DD (see text) and probed for collagen type I mRNA. The blot was then stripped and rehybridized with a PAL422(AP2) cDNA probe.

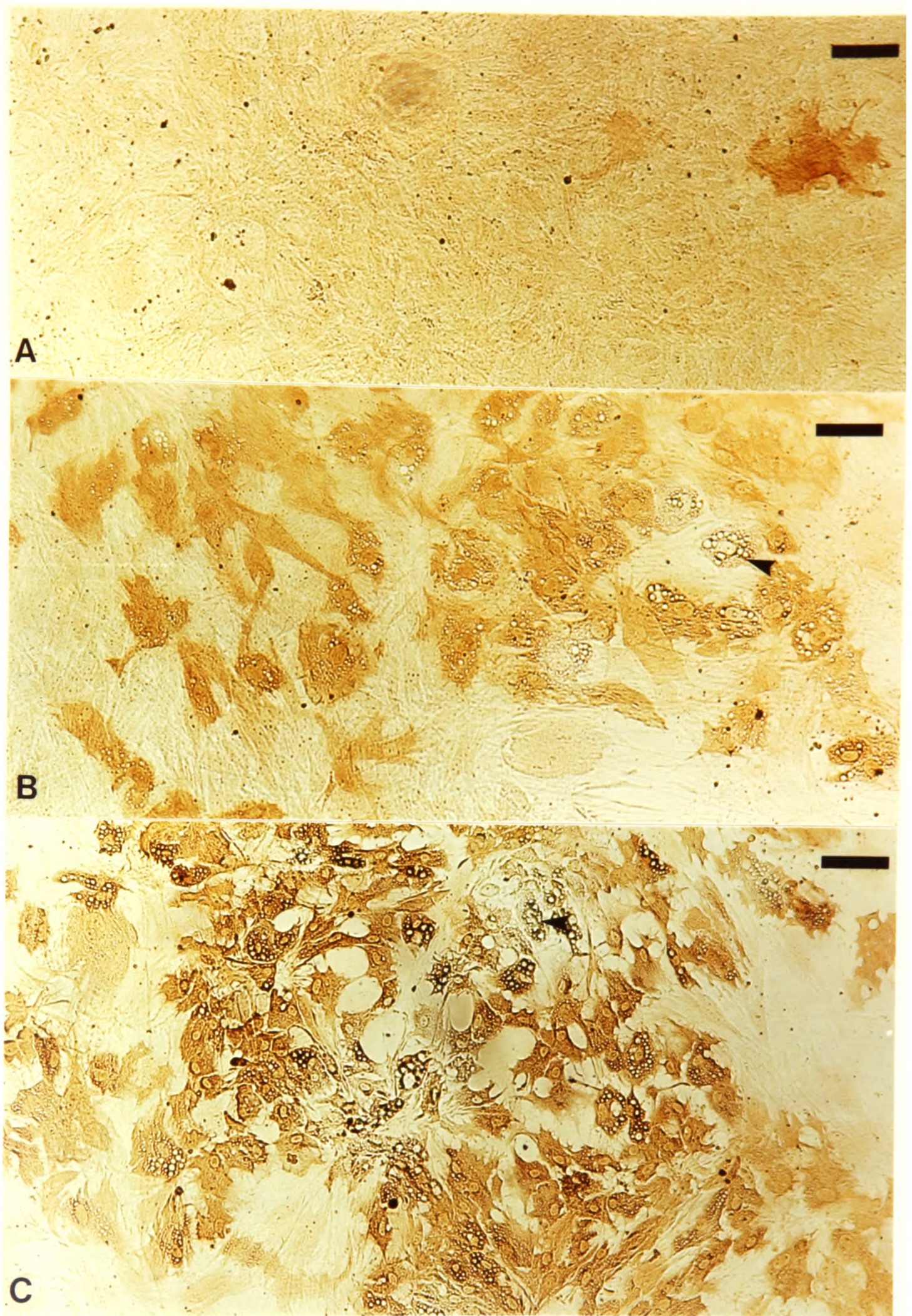


Plate 5.6.

Rat marrow stromal cells cultured under conditions 'CC' (A), 'CD' (B) or 'DD' (C), and stained using a polyclonal antiserum to localize PAL422(AP2) protein. Under conditions 'CC', PAL422(AP2) expression is rare. With conditions 'CD' (B) and 'DD' (C), expression of the protein is restricted to pre-adipocytes and adipocytes, most of which are characterized by cytoplasmic lipid vacuoles. Occasionally, cells with extensive cytoplasmic lipid vacuoles did not express the protein (arrowed).

CHAPTER 6. THE ISOLATION OF AN OSTEOGENIC MARROW STROMAL
CLONE BY LIMITING DILUTION.

6.1: INTRODUCTION.

When clonal populations of cells derived from embryonic calvarial bone surfaces which, on isolation, had osteoblast-like characteristics, were maintained in culture through extended passaging, markers of the osteoblast phenotype were not stable. Within individual populations the response to calcitropic hormones altered (Gutierrez et al, 1986) and there were qualitative changes in the collagen types produced (Grigoriadis et al, 1985). Close to primary culture, markers of the osteoblast phenotype showed greater stability. In one study, for example, markers of the osteoblast phenotype remained stable for the first 13 passages, but by the 22nd, cells had lost their osteoblastic character (Guenter et al, 1989). In order to have an unchanged population, therefore, cells need to be studied as close to primary culture as possible. In chapters 3 and 4 they have been expanded directly from single colonies in primary culture.

In order to reinforce the conclusion that there is a putative stromal stem cell present in rabbit marrow capable of giving rise to adipocytes, chondroblasts or osteoblasts (chapter 4), a more rigorous cloning technique involving the isolation of

single cells by limiting dilution was used prior to testing for expression of the osteoblastic or adipocytic phenotypes.

6.2: RESULTS.

6.2a: ISOLATION OF COLONIES BY LIMITING DILUTIONS.

96 microwells (0.2cm² surface area) were set up by limiting dilutions with less than 1.5 cells per well and were observed by phase contrast microscopy after 2 days, 6 days and 16 days. Single cells were visible in some wells after 48 hours but optical distortion made reliable observation difficult. By 6 days, discrete clusters were observed in 24 wells of which 4 contained more than 1 cluster. By day 16 some clusters had grown into colonies covering most of the surface area of the well whilst others remained as clusters. Six of the larger colonies were trypsinized and expanded by repeated passaging using a 1:2 split ratio. Of the 6 colonies isolated 3 gave a sufficient cell number ($\pm 4 \times 10^6$) to give aliquots for implantation and cryostorage. These were designated colonies 30, 31 and 32. The others failed to grow and were discarded.

6.2b: IN VIVO ASSAY OF OSTEOGENIC POTENTIAL.

10^6 cells from each colony were implanted intra-peritoneally, 1 chamber into each athymic MF1 mouse. After 55 to 58 days the chambers were removed, embedded in glycol methacrylate and sectioned for histological examination. A fibrous connective

tissue was seen in chambers containing tissue from colonies 30 and 32 whilst a mineralized matrix resembling bone and cartilage was seen in the chamber containing tissue from colony 31 (Plate 6.1a).

Aliquots of cells from colonies 30,31 and 32 were recovered and expanded to give a sufficient cell number to repeat the in vivo DC assay. 2 further chambers were implanted for each colony. Again, chambers with tissue from colonies 30 and 32 formed fibrous tissue. One further chamber with tissue from colony 31 formed fibrous tissue (Plate 6.1c) whilst the other formed a matrix resembling skeletal tissue (Fig 6.1).

Colony Number.	Diffusion Chamber.		
	1	2	3
Colony 30.	F.	F.	F.
Colony 31.	B.C.F.	F.	B.C.F.
Colony 32.	F.	F.	F.

Fig 6.1. Table summarizing the histological observations on DC's containing cells derived from Colonies 30, 31 and 32. B is a bone like matrix, C is a cartilage like matrix and F is a fibrous connective tissue. 10⁶ cells were implanted per chamber.

6.2c: IMMUNOHISTOCHEMISTRY.

Collagen types I, II, III and V were localized with goat anti rabbit antisera and a similar pattern of distribution was seen

to that described in chapter 3. Collagens I, III and V were seen in all sections whilst collagen type II was localized areas with morphological features of skeletal tissue.

When tested with BRL 12, a mouse anti rabbit bone specific keratan sulphate proteoglycan (Joyner et al, 1989; 1991), areas resembling mineralized tissue from colony 31 were positive whilst staining was not seen in chambers containing fibrous tissue alone (Plate 6.1b).

6.2d: EXPRESSION OF THE ADIPOCYTIC PHENOTYPE.

To test if cells from colony 31 were able to express features of the adipocytic phenotype, 25cm² tissue culture flasks were set up with α MEM, 10⁻⁷M Hc and 15% freshly prepared non-autologous RbP. Cells were inoculated into flasks in the range 7.5x10³ to 2.5x10⁵, and observed after 7 days. These observations are summarized in Fig 6.2.

Inoculation per Flask.	Observations on Live Cultures.
2.5	Confluent-fibroblasts.
1.25	Confluent-fibroblasts.
0.63	Confluent-fibroblasts.
0.31	Subconfluent-adipocytes.
0.075	Subconfluent-adipocytes.

Fig 6.2. Table summarizing observations made on live cultures of Colony 31 cells cultured in medium supplemented with 15% RbP and 10⁻⁷M Hc. Inoculation per flask is given x10⁵.

At higher seeding densities, $>6.25 \times 10^4$ cells per flask, cultures quickly became confluent. No adipogenesis was seen and cells appeared fibroblastic, acquiring a slightly granular appearance. At lower seeding densities, less than 3.1×10^4 cells per 25cm^2 tissue culture flask, tiny refractile cytoplasmic vacuoles with a typical pre-adipocytic morphology were seen after 7 days (Plate 6.1d). By day 11, clusters of cells were seen. Some appeared polygonal with a granular cytoplasm whilst others were larger, more rounded, with dense cytoplasmic lipid accumulations (Plate 6.1e).

6.3: DISCUSSION.

6.3a: CLONALITY OF MARROW STROMAL COLONIES.

The results provide further evidence that there is, present in the bone marrow a common precursor for osteoblasts and adipocytes. The major difference between this and previous chapters is in the method of isolation of clonal populations. In previous chapters, cloning rings were used to isolate single colonies as close to primary culture as possible. In this chapter a more rigorous cloning technique has been used, one widely used to isolate osteoblast-like populations regarded as clonal (Grigoriadis et al, 1988; Aubin et al, 1982), and the most rigorous available when planning this series of experiments. Even so, it was not possible to observe the development of a colony from a single cell, only from small clusters, thus it was not possible to be absolutely

certain that the populations formed were clonal, and they are regarded as clonal on the basis of overwhelming circumstantial evidence.

6.3b: RETROVIRAL MARKERS IN LINEAGE ANALYSIS.

A conclusive demonstration of clonality will await the application of new techniques such as the use of retroviral lineage markers. These techniques have been used with some success in other systems. In the study of the haemopoietic lineage, for example, bone marrow cells can be infected in vitro with retroviral vectors so that a proportion of the stem cell population becomes marked. Furthermore, the site of insertion will vary from stem cell to stem cell so that, by using restriction fragment analysis, each stem cell will have a unique , heritable label (Price, 1987). Bone marrow cells, for example, infected with a NEO vector, have been used to repopulate the marrow of lethally irradiated mice. Sequential analysis of host spleen, thymus and bone marrow indicated insertion of the vector into primitive pluripotent stem cells capable of giving rise to a range of cells of both the myeloid and lymphoid lineages (Dick et al, 1985).

Because of the requirement for a large quantity of DNA for biochemical analysis these techniques are unsuited to situations where the number of cells available is restricted. In these situations retroviral vectors have been employed in other ways. Vectors have been used which not only genetically

label the clone but also incorporate a gene which may be expressed. This gene may code a protein that can be recognised by histochemistry or by specific antibodies. the bacterial lac Z gene, for example, which codes the enzyme β -galactosidase, has been used to study lineage relationships in the nervous system (Price, 1987), and it is possible to speculate that the technique could be adapted to demonstrate the clonality of adherent marrow stromal colonies in vitro, or to identify osteoblast precursors in vivo.

6.3c: MULTIPOTENTIALITY OF CLONES 30, 31 AND 32.

Clone 31, unlike clones 30 and 32 was multipotential. Clonal cell lines have been reported which are capable of forming osteoblasts, chondroblasts, adipocytes and myoblasts (chapter 1.12), but have almost exclusively been derived from embryonic or neonatal tissue. Clone 31 is believed to be the first such clone derived from an older animal, and suggests that in rabbits, a pluripotential stem cell persists at least until adolescence.

Clearly there are marked differences between clones in terms of their differentiation potential in DC's, and future studies will be directed towards establishing if there are differences at a biochemical level in vitro. Preliminary studies using ^3H -Methionine labelling have indicated that the profile of proteins produced differs from clone to clone (results not given). These differences will be explored further using 2

dimensional gel electrophoresis to identify proteins specifically produced by cells capable of forming skeletal tissues.

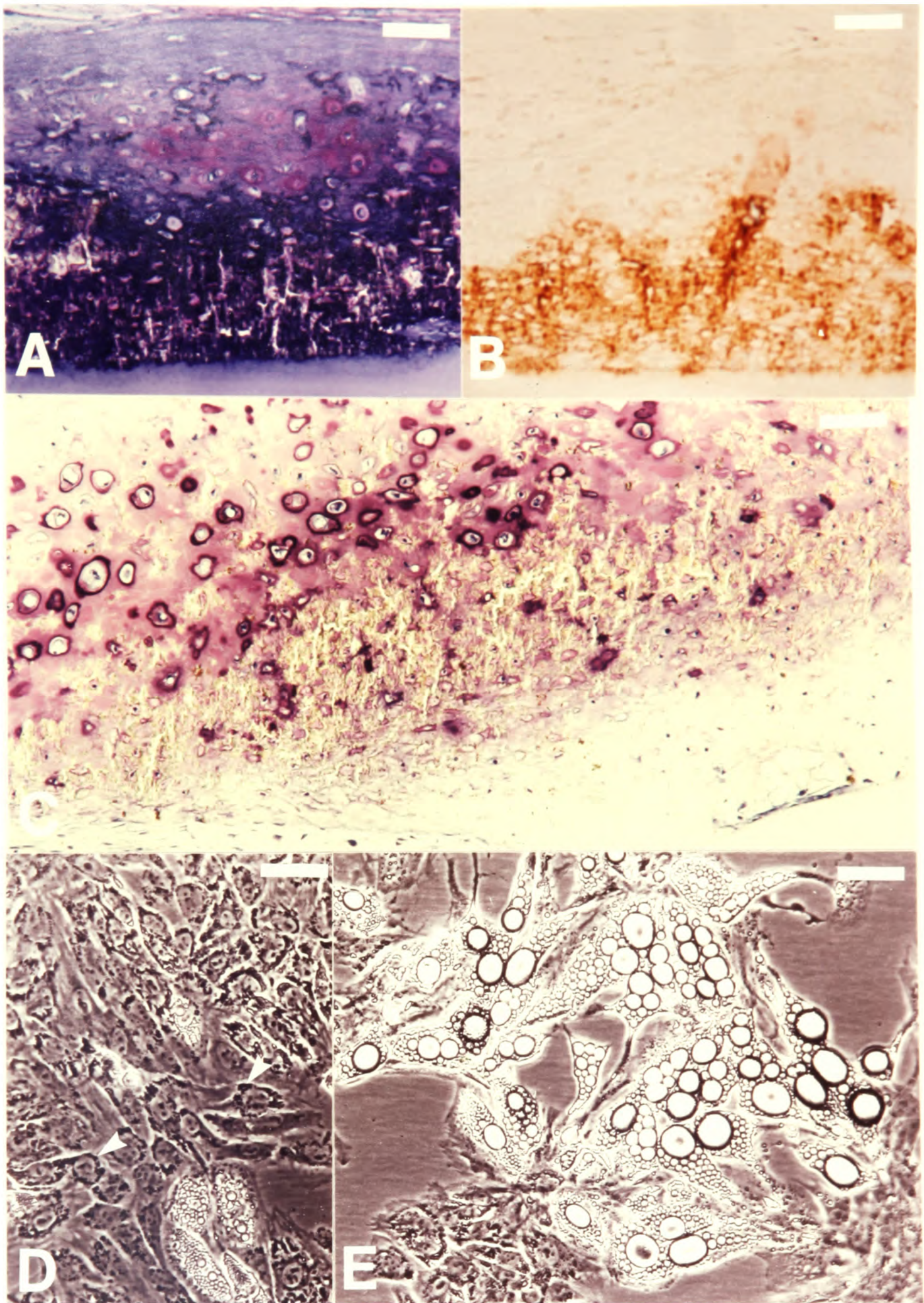


Plate 6.1.

5 μ m section of a DC containing cells from colony 31 stained with toluidine blue and showing a bone and cartilage like matrix (A). The same chamber was stained with monoclonal antibody BRL 12 to the rabbit bone specific proteoglycan keratan sulphate (B). Bar is 100 μ m.

The experiment was repeated and colony 31 cells formed a similar matrix in a second chamber (C). Bar is 100 μ m.

Cells from colony 31 were cultured in medium with 15% RbP at a seeding density of 3.13×10^4 per flask. After 7 days (D) they developed a granular pre-adipocytic cytoplasm (arrowed). E is the same flask on day 11 showing cells with cytoplasmic lipid filled vacuoles. Bar is 60 μ m.

CHAPTER 7. CONCLUDING REMARKS.

7.1: REVIEW OF AIMS AND OBJECTIVES.

The overall aim of this thesis has been to test a current hypothesis proposing a putative stem cell in adult marrow with the potential to differentiate into a number of stromal phenotypes including the osteoblast and osteocyte, the chondroblast and chondrocyte, and the adipocyte (chapter 1.3 and 1.15).

Populations of marrow stromal cells derived from young adult New Zealand white rabbits, each thought to be derived from a single cell, which have adipocytic characteristics in vitro, form a mineralizing matrix resembling bone and cartilage when implanted in vivo (chapters 3 and 4). This data complements earlier studies demonstrating that a stromal precursor derived from embryonic or neonatal tissue has the potential to differentiate in an osteoblastic, fibroblastic or adipocytic direction (section 1.10 and 1.12). Additionally, it was possible to conclude that the adipogenic and osteoblastic pathways diverge relatively late in the stromal lineage, and cells expressing features of the adipogenic phenotype still had the capacity to revert to a more primitive, proliferative state with a fibroblastic morphology, capable of forming bone in diffusion chambers.

Investigation of lineage relationships between cells of the marrow stromal system has been restricted by the lack of phenotype specific differentiation markers. There are several specific markers of extra-medullary fat differentiation, but very few studies have attempted to look at their expression in marrow adipocytes. A 14KDa P₂-like myelin protein PAL422(AP2) is a specific differentiation product of a number of extra-medullary cell lines, and it has been shown here to be a useful marker of adipogenic differentiation in rat marrow cell cultures (chapter 5).

Data presented in this thesis is relevant to understanding the complex relationship between marrow fat and bone tissue, a relationship that has implications for the pathology and physiology of bone and blood cells.

7.2: MARROW FAT AND OSTEOPOROSIS.

The inter-relationship between bone remodelling and medullary fat is of great interest because of its importance in the aetiology of widespread human disease. Histomorphometric studies on bone biopsies have demonstrated that, in primary osteoporosis as well as that associated with old age, trabecular bone is replaced by marrow fat, and this process is regarded as irreversible (Burkhardt et al, 1987; Meunier et al, 1971). These results are supported by animal experiments. A decrease in the amount of bone was accompanied by an increase in marrow fat volume in ovariectomized dogs (Martin

et al, 1990). In growing and mature rabbits, administration of glucocorticoids increased marrow fat volume whilst decreasing trabecular bone volume (Kawai et al, 1985; Wang et al, 1977).

7.3: A SPECULATIVE ROLE FOR MARROW FAT.

The marrow changes observed in osteoporosis remain unexplained. It is not known if, for example, they are a cause or an effect of the changes in trabecular bone. A speculative answer is that the formation of marrow fat is secondary to the bony changes, and that the function of the marrow adipose tissue is to fill redundant marrow space previously occupied by bone or haemopoietic tissue. Some evidence comes from Bianco et al (1988b), who studied bone marrow biopsies from patients with acute non-lymphoid leukaemia. These patients were in an aplastic phase, shortly after completion of a course of chemotherapy. This represented a unique situation, as the marrow had been rapidly and temporarily depleted of haemopoietic cells, but not stromal cells (Islam et al, 1980). In this situation the marrow, depleted of haemopoietic cells was transiently repopulated by fat cells, some of which had membrane bound AP activity, before the re-development of a functional haemopoietic marrow.

7.3: RED AND YELLOW MARROW.

It has been estimated that a 70Kg adult will have 1200-1500g of red marrow out of a total marrow space of 2600cc to 4000cc.

In a 15Kg child, with a marrow space of 1600cc, there is 1000g to 1400g of red marrow (Bierman, 1961; Custer, 1949). Thus, despite large differences in the amount of marrow space available, the volume devoted to haemopoiesis remains remarkably constant, and, in the adult, the remainder is filled with fatty marrow. This implies that the amount of red marrow is highly regulated, and the ratio of red to yellow marrow is tightly controlled.

The amount of haemopoietic marrow is partially determined by environmental factors. Under prolonged haemopoietic stress, for example, secondary to the haemolytic anaemias, leukaemias, infection or altitude, yellow marrow may revert to red marrow (Bierman, 1961), but the cellular events involved remain unknown.

Again, it could be argued that the role of the marrow adipocytes was that of a space maintainer, and it is possible to speculate that the ratio of red to yellow marrow represented an ill defined homeostatic mechanism for the maintenance of a highly regulated volume of erythropoietic marrow.

7.4: BONE MATRIX CONSTITUENTS AND ADIPOGENESIS.

Very few studies have looked at the effect of bone matrix constituents on pre-adipocyte cell lines, extra-medullary or medullary adipocytes. One exception is TGF- β , which, although

a minor bone matrix constituent, is a growth factor of considerable importance and may be released in an active form during normal bone turnover (Pfeilschifter and Mundy, 1986; Mundy et al, 1990). TGF- β promotes osteogenic differentiation in vitro in isolated osteoblast-like populations and osteosarcoma-derived cell lines (Canalis et al, 1989), and in vivo in periosteal stromal tissue (Joyce et al, 1990). TGF- β inhibited adipogenesis in pre-adipocytic cell lines (Ignatz and Massague, 1985; Sparks and Scott, 1986), and porcine extra-medullary adipose tissue (Richardson et al, 1989). Preliminary results suggest that, at a concentration of 0.1ngml^{-1} , TGF- β inhibits adipogenesis in rabbit marrow cultures in vitro (work in progress-results not given). Even with the limited amount of evidence available, it is possible to hypothesize that there are, within the bone matrix or produced by local cells, growth factors capable of promoting osteogenic differentiation whilst inhibiting adipogenic differentiation. Their significance, however, remains unknown.

7.5: FUTURE DIRECTIONS.

Finally, I should like to speculate about factors that promote differentiation of marrow stromal precursors in an adipogenic direction.

Differentiation to form osteoblasts might, for example, require specific bone matrix proteins with growth factor activity. In the absence of these factors, differentiation

cannot proceed in an osteogenic direction and marrow adipocytes are formed, thus, differentiation into adipocytes represents a 'default ' differentiation stage into which precursors differentiate in the absence of alternative environmental stimuli. Such a scheme would explain the switch to a fatty marrow secondary to the loss of the trabecular structure in osteoporosis. In this situation, the trabecular surface area is considerably reduced and the stromal precursors are less likely to come in contact with the bone matrix, thus they are less likely to be subject to those influences promoting differentiation in an osteogenic direction.

A similar argument could be applied to the switch that can occur between red and yellow marrow. Erythropoietic stimuli might act on stromal precursors to promote differentiation in the direction of a marrow stroma capable of supporting haemopoiesis. Absence of these factors leads to a fatty marrow.

Investigation of the effect of specific growth factors found in the bone matrix, and specific erythropoietic factors on clonal populations of adipogenic bone marrow cells is, therefore, a subject of great interest, and one which represents a considerable challenge for the future.

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