THE RAT BONE MARROW STROMAL CELL OSTEOGENIC SYSTEM:
CHARACTERIZATION OF SUBPOPULATIONS, FRACTIONATION,
AND EFFECTS OF PDGF

by

Alexandra Lenore Herbertson

A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
Graduate Department of Dentistry
University of Toronto

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ABSTRACT

The objective of this thesis was to better define and characterize the heterogeneous cellular environment of the rat bone marrow (RBM) stromal cell osteogenic system. I began by identifying and quantifying the major subpopulations present when RBM stromal cells are grown under conditions stimulating bone formation, i.e., supplementation with dexamethasone (dex). As expected, dex significantly increased the number of alkaline phosphatase (AP) positive colonies and von Kossa positive bone nodules, but concomitantly stimulated the number of macrophage colonies, while reducing the number of hemopoietic cells expressing leukocyte common antigen. Since the presence of these multiple cell types may complicate determination of mechanisms of regulation of the osteoprogenitors, we next investigated methods for purification of the osteoblast lineage cells. Flow cytometric sorting performed on the basis of AP expression resulted in $AP_{\text{high}}$ and $AP_{\text{low}}$ fractions of cells: within the $AP_{\text{high}}$ fraction, the number of AP-positive colonies and bone nodules were significantly enriched. Adipocyte and macrophage colonies were significantly depleted in the $AP_{\text{high}}$ fraction and consistently enriched in the $AP_{\text{low}}$ fraction of cells.

Finally, I investigated the effect of PDGF, a factor produced in abundance by macrophages, in fractionated and unfractionated RBM stromal populations. While stimulating proliferation and differentiation of the macrophage and adipocytic lineages, PDGF had
dose- and time-dependent biphasic effects on bone nodule formation, stimulating at low doses and at early stages of osteoblast differentiation but inhibiting at high doses and later; suggesting that PDGF has pleiotropic effects on bone metabolism, and that its effects on bone might be direct on osteoblast lineage cells.

Together, my data validate methods for enrichment of osteoprogenitors in RBM stromal cultures and provide a stronger basis for investigating the nature of their regulation by hormones, cytokines, and growth factors.
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I also wish to acknowledge the financial support of the Medical Research Council of Canada through their Dental Fellowship program, and the support of the Harron Trust Fund, without which this work would not have been possible.

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<td>αNBE</td>
<td>α-naphthyl butyrate esterase</td>
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<td>AP</td>
<td>alkaline phosphatase</td>
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<td>BM</td>
<td>bone marrow</td>
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<tr>
<td>CDR</td>
<td>colour development reagent</td>
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<tr>
<td>CFU-F</td>
<td>fibroblastic colony-forming unit</td>
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<td>CFU-O</td>
<td>osteoprogenitor colony-forming unit</td>
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<td>CFU-ST</td>
<td>stromal colony-forming unit</td>
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<td>d.</td>
<td>day(s)</td>
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<tr>
<td>dex</td>
<td>dexamethasone</td>
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<tr>
<td>ED2</td>
<td>monoclonal antibody specific against rat macrophage cells</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>GAM</td>
<td>goat anti-mouse</td>
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<td>GM-CSF</td>
<td>granulocyte/macrophage colony-stimulating factor</td>
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<td>HPR</td>
<td>horseradish peroxidase</td>
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<tr>
<td>hr.</td>
<td>hour(s)</td>
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<td>IL-1</td>
<td>interleukin 1</td>
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<tr>
<td>IGFBP</td>
<td>insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>LCA</td>
<td>leukocyte common antigen</td>
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<tr>
<td>LNGFR</td>
<td>low-affinity nerve growth factor receptor</td>
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<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mAB</td>
<td>monoclonal antibody</td>
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<tr>
<td>M-CSF</td>
<td>macrophage colony stimulating factor</td>
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<tr>
<td>MRC OX-1</td>
<td>monoclonal antibody against leukocyte common antigen</td>
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<td>MRC OX-22</td>
<td>monoclonal antibody against rat lymphocytes</td>
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<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>PGE₂</td>
<td>prostaglandin E₂</td>
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<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
</tr>
<tr>
<td>RBM 211.13</td>
<td>monoclonal antibody specific against rat alkaline phosphatase</td>
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<tr>
<td>RBM</td>
<td>rat bone marrow</td>
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<tr>
<td>RECA-1</td>
<td>monoclonal antibody specific against rat endothelial cells</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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CHAPTER 1

Introduction
1.1 General Introduction

Bone marrow (BM) is a complex tissue consisting of many different cell types with distinct functions existing together in a coordinated, regulated and inter-dependent fashion. BM can be divided into two broad compartments: the hemopoietic cells, a primarily nonadherent population in cell cultures, and the stromal cells, primarily adherent in cell cultures. Regulation of mammalian hemopoiesis is dependent on a microenvironment composed of a variety of hemopoietic cells and stromal cells and their extracellular matrix (Dexter, 1982; Ross et al., 1991; Long, 1992). It also appears that stromal cell growth and development, including those of osteogenic cells, is regulated by the local microenvironment, as hemopoietic cells can produce cytokines known to affect the growth and differentiation of stromal cells, and BM stromal cells can release factors affecting other stromal cells (Ogiso et al., 1991; Yanai et al., 1991; Zhang et al., 1991; Sasaki and Hong, 1993).

1.2 Non-Osteogenic Stromal Cell Types

It has been recognized that BM stroma contains a mixed population of cell types (Dexter, 1982), but many published reports remain focused on only one or a few of the cell types in the culture. In mouse hemopoietically active long-term BM stromal cultures, the cell populations have been identified as: 21 ± 2% fibroblastoid, 3 ± 0.3% endothelial, 26 ± 3% adipocytic, and 49 ± 1% macrophage (Hauser et al., 1995), accounting for essentially all (99%) of the stroma. In rat bone marrow (RBM) stromal cell cultures, little is known about the subpopulation make up, except that in 10 - 12 d. primary cultures the colony distribution was found to be 85% fibroblast-like, 10.3% macrophage/dendritic/granulocyte, and 2.5% endothelial (Simmons et al., 1991). Maniatopoulos et al. (1988) also noted in primary RBM stromal cultures the presence of small, mononuclear (hemopoietic) cells (Maniatopoulos et
al., 1988); the repeated medium changes in the 7 d. primary culture period in Maniatopoulos' method is used to reduce hemopoietic contamination and cellular debris while expanding the numbers of CFU-F. Even in the culture conditions selected to maximize osteogenesis, only a proportion of the cells in RBM stromal cell cultures are osteogenic, and the presence of other morphologically and histochemically/immunohistochemically distinguishable cell types has been reported (Malaval et al., 1994). The multiple cell types present reflect, at least in part, the multiple lineages in marrow stroma, including also multipotential mesenchymal stem cells with the ability to differentiate along various pathways. As the presence of multiple subpopulations complicates the elucidation of cell-cell interactions within osteogenic stroma, documentation of which cells are present and in what quantities is needed to unravel the interactions. A brief summary follows of the cell types that have been reported in BM stromal cell cultures (of a variety of species).

*Fibroblasts*

This group includes definitive fibroblasts involved in forming the supporting connective tissue of marrow. However, the designation of "fibroblast" has also been used as a general category of BM stromal cells which are undefined or undifferentiated. The term colony forming unit - fibroblast(oid) or CFU-F has been used in reference to colonies which can include fibroblasts, adipocytes, endothelial cells, chondro- and osteoprogenitor cells, smooth muscle cells, reticular and reticular-fibroblastoid cells, and mesenchymal stem cells often in combination with macrophages, mast cells, myeloid cells, and hemopoietic stem cells (Wang et al., 1990; Benayahu et al., 1991; Simmons et al., 1991). Another term used is colony forming unit stromal or CFU-ST for stromal or adherent cells (McIntyre and Bjornson, 1986). Assessments of results from experiments designating colonies as CFU-F can be difficult to interpret in light of this potential variety in cell composition. For example, PDGF is known to increase growth of CFU-ST (McIntyre and Bjornson, 1986), but whether this is a response from multiple populations of cells or just one cell type is not
known. A monoclonal antibody (mAb), STRO-1, was developed which (in combination with a glycophorin A negative screen) sorts CFU-Fs from other human BM cells (Simmons and Torok-Storb, 1991). Cells sorted on this basis (glycophorin-negative, STRO-1-positive) give rise to fibroblasts, smooth muscle cells, and adipocytes which results in an adherent layer able to support hemopoiesis (Simmons and Torok-Storb, 1991). Osteoprogenitors capable of differentiating into functional osteoblasts are also present in adult human BM-derived STRO-1-positive CFU-F (Gronthos et al., 1994). Taken together, the data suggest that STRO-1 may be associated - although not exclusively - with stromal mesenchymal stem cells. More recently, Joyner et al. (1997) have also isolated a mAb (HOP-26) reactive against human CFU-F, including osteoprogenitors (Joyner et al., 1997). Amongst a series of mABs developed against RBM stroma, ST3 and ST4 are reactive against fibroblasts (Sullivan et al., 1989) but ST3 labels primarily marrow fibroblasts and ST4 labels primarily fibroblasts in other tissues, suggesting that the fibroblastic cells of the marrow are different from those of non-hemopoietic tissues (Sullivan et al., 1989). Treatment with ST3 and complement prior to culture decreases CFU-F (and hemopoietic colony formation) (Sullivan et al., 1989). While the development of mAbs has aided in identification of fibroblastic cells in BM, many cells are labelled fibroblastic for want of other identifying characteristics, and the term undoubtedly encompasses a variety of cell types.

**Endothelial cells**

Endothelial cells have been frequently, although not consistently, identified in BM cell cultures. Isolated endothelial cell colonies can be obtained from mouse BM with low plating numbers of less than 20 BM stromal cells/35mm dish in medium supplemented with GM-CSF (10 U/l) (Wang and Wolf, 1990). Mouse endothelial cells have dendritic cytoplasmic processes, round nuclei, and overlapping cytoplasms in confluent cultures (Yanai et al., 1991). Histo- and immunohistochemical identification of endothelial cells has historically posed a problem. Endothelial cells, like macrophages, will take up low density
lipoprotein. In mice, endothelial cells bind Ulex europaeus 1 lectin (Yanai et al., 1991). Factor VIII antigen may or may not be detected in mouse endothelial cells (Wang et al., 1990; Yanai et al., 1991). Polyclonal antibodies against von Willebrand factor are not endothelial cell-specific and do not identify all endothelial cells (Duijvestijn et al., 1992). While alkaline phosphatase (AP) activity has been reported in endothelial cells, high activity appears to be associated with isolated brain capillary endothelial cells in vivo, while endothelial cells lining blood vessels elsewhere do not contain significant levels (Meyer et al., 1990). The recent development of mAbs specifically recognizing endothelial cells in mouse marrow (Hasthorpe et al., 1990) and in rats (RECA-1 = rat endothelial cell antigen) (Duijvestijn et al., 1992) has made definitive identification of these cells easier.

Reticular cells

Reticular cell is a histomorphometric term for the adventitial cell which forms the outer layer surrounding the sinuses in the BM; the reticular cell is thought to send out sheet-like extensions containing reticular fibres to form a three dimensional network (reticulum) of cytoplasmic extensions throughout the marrow (Westen and Bainton, 1979). The reticular cell is fibroblastic in appearance, is characterized by its membrane-bound AP, and associates mainly with granulocytic precursors (Westen and Bainton, 1979). Antibodies against low-affinity nerve growth factor receptor (LNGFR) label stromal reticular cells with dendritic features in fresh smears and in formalin-fixed, paraffin embedded human BM (Cattoretti et al., 1991). These LNGFR positive cells are also positive for AP, reticulin, collagen type III, and are negative for neural, endothelial, and leukocyte markers (Cattoretti et al., 1991). LNGFR positive cells have an oval nucleus, a scanty cytoplasm with long dendrites that intermingle with hemopoietic cells, line the abluminal side of sinus endothelial cells, and provide the scaffold for the hemopoietic marrow (Cattoretti et al., 1991). The reticular stromal cells appear in the fetal BM before the hemopoietic activity begins, originate from the vessel adventitia, and radiate in the BM cavity (Cattoretti et al., 1991). Barrier cell is
another term for a reticular cell described by Weiss and Geduldig (1991) in a microscopic histological study of mouse BM. Barrier cells run from the bone-lining layer to subosteal blood vessels and appear associated with all stages of hemopoiesis (Weiss and Geduldig, 1991). Recent works have described cell correlates in vitro to the reticular cell in vivo. Anti-LNGFR antibodies label 7 - 11% of the fibroblastoid population in 4 week-old long-term human BM cultures, suggesting that these cells are reticular cells (Cattoretti et al., 1991). In another study, LNGFR positive cells with multiple, long dendritic processes were similarly found in long-term human BM cultures; while few were seen in 2 week old cultures, their numbers increased up to 6 weeks (Wilkins and Jones, 1995). Since AP is a common cytochemical marker of both 'reticular' cells and preadipocytes, it has been suggested that reticular cells may convert to adipocytes when marrow cellularity abruptly decreases (Bianco et al., 1988). In murine BM cultured in methylcellulose/plasma clot, white blood cell conditioned medium, dexamethasone (dex) and serum, extensive branching colonies developed which were called reticulofibroblastoid and were thought to become adipocytic by d. 14 of culture (Ross et al., 1991). It is unclear whether these cells represent a differentiation stage in a preadipocytic lineage, or a multipotential stem cell lineage, or mixed colonies derived from multiple lineages as whole marrow cell cultures were used. However, the authors felt that reticulofibroblastoid cells are a distinct lineage on the basis of morphology, adipocyte formation, and production of collagen type IV, and with their long branching arms may be important in setting up compartments for hemopoiesis in mouse BM (Ross et al., 1991).

**Adipocytes**

Adipocytes are a well-known component of BM. In long-term BM cultures, the presence of fat cells is taken as a marker of a hemopoietically productive culture (Ross et al., 1991). Adipocytes have been described in combinations with many other populations of marrow cells, e.g., reticulofibroblastoid cells as previously mentioned (Ross et al., 1991). Bennett
and others (1991) described stromal colonies from rabbit BM with fibroblastic morphology that had differentiated in an adipocytic direction, but that subsequently reverted to a more proliferative stage and differentiated along the osteogenic pathway (Bennett et al., 1991). These, and other data, have given rise to the notion of a bipotential adipocyte-osteoblast progenitor or adipocyte-osteoblast switch. For example, long-term murine marrow culture stromal clone (BMS2) has been produced which can differentiate into adipocytes; while BMS2 has been used to examine the regulation of preadipocytic cells (Gimble et al., 1991), it also demonstrates osteogenic characteristics (Dorheim et al., 1993). The data are interesting, but further work will be needed to fully characterize the nature of the adipocyte-osteoblast phenotypic transformation (see, for e.g., (Gimble et al., 1996; Aubin and Heersche, 1997), for reviews).

Smooth muscle cells
In long-term human BM cultures, fibroblastoid cells expressing α-smooth muscle actin have also been described (Wilkins and Jones, 1995). These cells appear at approximately 2 weeks, and form a majority of the stromal population within 4 - 6 weeks, under at least some culture conditions (Wilkins and Jones, 1995). In long-term adult human BM cultures, stromal myoid components were also present when the stromal cells were cultured without hydrocortisone and horse serum; the presence of the latter two additives actually inhibited myoid cell growth and development (Bonanno et al., 1994).

Hemopoietic Cells
Hemopoietic cells exist in intimate association with stromal cells in BM in vivo and in vitro. To remove the majority of well-differentiated hemopoietic cells when culturing stromal cells, centrifugation or adherence techniques are commonly used. Hemopoietic stem cells in mouse BM can be enriched by adherence to tissue culture plastic, but it is not known if the cells are adhering to the plastic or to a stromal cell (Kiefer et al., 1991). In mouse BM,
hemopoietic stem cells are present at a frequency of 1 in 25,000 in the adherent cell layer (Kiefer et al., 1991), and their subsequent differentiation is influenced by the existing culture conditions. Hemopoietic cell types described in BM stromal cultures include macrophages (Dexter, 1982; McIntyre and Bjornson, 1986; Wang and Wolf, 1990; Keller et al., 1993; Wilkins and Jones, 1995), mast cells (McIntyre and Bjornson, 1986), dendritic cells (Giesler et al., 1991; Miyagi and Kimura, 1993), osteoclasts (Udagawa et al., 1990), granulocytes (Simmons et al., 1991), and megakaryocytes (Briddell et al., 1992).

Macrophages have long been known as a component of long-term BM stromal cultures (Dexter, 1982). In mice, macrophages are frequently seen in BM stromal cell cultures and isolated macrophage colonies can be obtained at the low plating density of 20 or less BM stromal cells/35mm dish using media supplemented with GM-CSF at 10 U/l (Wang and Wolf, 1990). Mouse macrophages can be identified with mAbs or histochemical stains such as berberine sulphate or α-naphthyl butyrate esterase (αNBE) (Kanemoto et al., 1991). In human BM cultures, macrophages have been identified in the adherent layer (Keller et al., 1993; Wilkins and Jones, 1995). While one study found a rather homogeneous macrophage population (Keller et al., 1993), another identified two types of macrophages (Wilkins and Jones, 1995). One type had a rounded morphology and abundant phagocytosed debris, while the other type had cells that were elongated and flattened, displayed several short cytoplasmic processes, and lacked phagocytosed debris (Wilkins and Jones, 1995). The second type of macrophage was seen only in more mature cultures (Wilkins and Jones, 1995). The number of macrophages present varied significantly between long-term BM culture isolates, and did not correlate with the number of hemopoietic foci present (Wilkins and Jones, 1995).
1.3 Osteogenic Stromal Cells

Friedenstein and colleagues first demonstrated that BM stroma contains cells with the capacity to form bone when they are transplanted in vivo in diffusion chambers (Friedenstein et al., 1968). This has been confirmed repeatedly in vivo with BM cells in diffusion chambers (Ashton et al., 1984; Owen, 1988) and, more recently, conditions have been established in which bone formation is observed in vitro in cultures of BM stromal cells isolated from several species. Tibone first reported bone production in vitro by BM stromal cells in aspirated femoral marrow from adult rabbits (Tibone and Bernard, 1982). Since then, other nonclonal animal models of BM osteogenesis, with bone nodules as the terminal product, have also been characterized and optimized for more reproducible and more copious osteoblastic differentiation. Under culture conditions previously established to support and maximize osteogenesis in cultures of folded chick periostea (Tenenbaum and Heersche, 1982) and osteoblastic cells derived from calvaria (Bellows et al., 1986), bone nodule formation in cultures of stromal cells has been seen in young-adult rat femoral BM (Maniatopoulos et al., 1988), chick embryo tibial and femoral BM stroma (Kamalia et al., 1992), neonatal pig tibial and femoral BM stromal cells (Thomson et al., 1993), young adult mouse femoral BM stroma (Van Vlasselaer et al., 1993), and adult human BM (Vilamitjana-Amedee et al., 1993). However, optimal conditions for the formation of discrete bone nodules in these different BM stromal cultures differ, likely reflective of species or donor age differences. For example, culture conditions similar to those used in rat stroma (Maniatopoulos et al., 1988) result in mineralization without nodule formation in adult human stroma (Cheng et al., 1994). This may in part be a reflection of the age of the BM donor, as age has been reported to affect the yield of nodules and/or mineralized matrix (see discussion in (Vilamitjana-Amedee et al., 1993)), and discrete bone nodules have been reported in young adult human BM stroma (Vilamitjana-Amedee et al., 1993). The importance of species differences is obvious when looking at dex as an additive: $10^{-8}$ M dex
increases the apparent number of osteoprogenitor cells which give rise to bone nodules by 5-50-fold in RBM stromal cells (Aubin et al., 1990) while $10^{-7}$ M dex has been determined to be optimal for mineralization in a human system (Cheng et al., 1994), and concentrations up to $10^{-10}$ M significantly reduce bone nodule formation in mouse BM stroma (Falla et al., 1993). As well, mouse BM stroma needs to be plated at higher densities to obtain CFU-F numbers comparable to those in the rat system (unpublished observations), resulting in at least some attempts to develop strategies for enrichment of osteoprogenitors after general isolation protocols (Falla et al., 1993).

The mineralized bone nodules formed in these mixed stromal population systems have morphological characteristics similar to authentic woven bone with its associated cells. Specifically, in RBM stromal cultures, a bone nodule has rows of cuboidal cells with oval nuclei and prominent nucleoli, well-developed rough endoplasmic reticulum, and prominent Golgi (Satoumura and Nagayama, 1991) on the surface, separated from the mineralized material by an osteoid-like matrix; cells resembling osteocytes are embedded in the mineralized matrix (Maniotopoulos et al., 1988) and some cells protrude cell processes toward neighbouring cells through the extracellular matrix (Satoumura and Nagayama, 1991). X-ray microanalysis and X-ray diffraction respectively have been used to show the presence of Ca and P in the matrix, in the form of hydroxyapatite (Maniotopoulos et al., 1988). RBM stromal cells express PTH receptors (Zhang et al., 1995), and the cells associated with nodules exhibit high AP activity (Maniotopoulos et al., 1988) and produce type I collagen and SPARC/osteonectin, along with the non-collagenous bone proteins osteocalcin and bone sialoprotein (Maniotopoulos et al., 1988; Kasugai et al., 1991; Malaval et al., 1994).
1.4 Stromal Cell Interactions

*General cell-cell interactions*

While different BM stromal cells are known to interact, in most cases the mechanism of the interaction is not clear, although multiple cytokines and growth factors produced by different cell types present are likely to play roles (see Section 1.6 below). For example, adipocytes can stimulate the growth of endothelial cells, while endothelial cells can inhibit the growth and development of preadipocytes (Yanai et al., 1991). Rat marrow stromal fibroblast-like cells produce a number of osteotropic factors that at low concentrations stimulate, or at high concentrations inhibit, calvarial osteoblast growth and there is evidence for the involvement of insulin-like growth factors (Zhang et al., 1991). Rat skin fibroblasts and periodontal ligament fibroblasts release soluble factors into their conditioned medium, including prostaglandins, that inhibit osteoblast differentiation in BM stroma (Ogiso et al., 1991). On the other hand, bone formation is increased in stromal cell cultures when conditioned medium from rat calvaria cells is added, suggesting that mature osteoblasts produce a paracrine growth factor that can stimulate the differentiation of osteoblasts from precursor cells (Hughes and McCulloch, 1991). It has also been suggested that factors produced by BM cells may affect other BM stromal cells that are distant from the factor source; healing RBM produces a growth factor activity with a preferential effect on osteogenic cells (Bab et al., 1988). As well, marrow ablation in rat long bones induces an increase in osteogenesis in distant skeletal sites and the systemic osteogenic response is thought to be mediated by circulating factors produced by the healing marrow (Bab et al., 1988; Gazit et al., 1990; Bab et al., 1992).

*Limiting dilution studies*

In rat calvaria and RBM stromal bone model systems, the number of nodules or colonies capable of forming bone can be counted for an assessment of osteoprogenitor numbers.
recoverable from the tissue and able to undergo differentiation in vitro under certain test conditions (e.g., rat calvaria (Bellows and Aubin, 1989); RBM stroma (McCulloch et al., 1991)). Limiting dilution analysis was used to investigate the differentiation process of these restricted monopotential osteoprogenitor cells in these primary cultures. In rat calvaria cell populations, limiting dilution indicated a "single-hit" phenomenon, i.e. that only one cell type was limiting for nodule formation; the data are consistent with the limiting cell type being the osteoprogenitor itself, and with such cells being present at a measurable but low frequency (<1%) of the total population under standard isolation and culture conditions (Bellows and Aubin, 1989). In contrast, in RBM stromal secondary populations expression of neither total CFU-F nor the osteoprogenitor subfraction (CFU-O) follows a linear relationship in limiting dilution analysis until very high cell densities are reached, suggesting cooperativity of cell types within the population and a multi-target phenomenon (Aubin et al., 1990). In multi-target events, the response (production of a bone nodule) is observed only when at least one cell of each of $m$ categories is present in the sample (Lefkovits and Waldmann, 1979), i.e., that at least two cell types are limiting for bone nodule formation in RBM stroma. When a constant number of non-adherent (hemopoietic) BM cells was added back to the limiting dilution analyses of the stromal cells, about 1/100 CFU-O were measured and their expression now followed a linear, single-hit relationship (Aubin et al., 1990). Endothelial cells or endothelial cell conditioned medium had similar effects on CFU-O expression (Aubin et al., 1990). Similarly, formation of bone nodules in vitro from murine marrow CFU-F plated at low density requires the presence of irradiated feeder cells, and PDGF, IL-3, and EGF appeared not to be able to substitute for feeder cells, leading to the suggestion that another as yet unidentified factor produced by the irradiated cells was required (Friedenstein et al., 1992). These data suggest that stromal osteogenesis in RBM may be under the regulation of other cells in the BM which can exert either stimulatory or inhibitory activities.
1.5 Purification of osteoblasts from stroma

Given the multiple subpopulations reported to be present in stroma, methods for enriching or separating the osteogenic subpopulations is desirable. Several strategies have been used to produce enriched osteoblast populations from mixed stromal populations, but these can be grouped into adherence techniques or antibody-based techniques.

*Adherence characteristics*

While nonadherence to plastic has been reported to be characteristic of the 'majority' of osteoprogenitor cells in mouse (Falla et al., 1993) and human (Long et al., 1990; Long et al., 1995) BM, most BM stromal nodule systems rely on (slow) adherence to plastic for isolation of osteoblastic cells. There is comparable osteoprogenitor plating efficiency in control and nonadherent (1 hr. at 37° C in a tissue culture flask) mouse marrow cultures (Falla et al., 1993) and in control and nonadherent (24 hr. at 37° C in a tissue culture flask) primary RBM cultures (Scutt and Bertram, 1995). In the original descriptions of osteogenic rat (Maniatopoulos et al., 1988) and chick (Kamalia et al., 1992) BM, stromal cells were allowed to attach for 24 hr. prior to the first medium change (and medium is changed completely 3 times per week thereafter). The frequent medium changes are thought to reduce hemopoietic contamination (Maniatopoulos et al., 1988; Kamalia et al., 1992) while expanding the CFU-F (Maniatopoulos et al., 1988), of which osteoprogenitors are a subgroup; however, significant numbers of hemopoietic cells continue to be present into the secondary culture even with this protocol (Herbertson and Aubin, 1995). In protocols described for adult human BM (Cheng et al., 1994), mouse BM (Van Vlasselaer et al., 1993), and porcine BM (Thomson et al., 1993), longer adherence times are used, and the medium is changed only after 5 - 7 d. of primary cultures. Scutt and colleagues have reported recently that PGE2 can induce cells in the nonadherent population in RBM to form adherent CFU-F, a proportion of which are osteoprogenitors which give rise to osteoblasts
which synthesize bone-like tissue (Scutt and Bertram, 1995). In these studies, the action of PGE$_2$ was shown to be dependent on the glucocorticoid (exogenously added dex) levels present in the cultures, suggesting that both time and cytokine conditioning are factors to be considered in the establishment of protocols for enrichment of osteoblastic populations.

**Antibodies**

The finding that cells at different stages of the osteoblast lineage express antigenic determinants unique or distinctive to these stages is beginning to be useful for identification and isolation of osteoblastic cells at various stages of differentiation (Aubin and Turksen, 1995). Thus, antibodies may be useful not only for identification of diverse cell lineages within stromal populations, but also to characterize differentiation stages within the osteoblast lineage and to fractionate populations based on epitope expression. Of antibodies raised against osteoblastic cells and reported to date, those recognizing the more mature cells in the lineage (osteoblasts and osteocytes) have been more commonly found than those recognizing less mature cells. Enrichment of relatively mature osteoblastic cells has been reported. Van Vlasselaer and colleagues used immunopanning with mAbs to first deplete populations of hematopoietic cells, then used flow sorting with Sca-1 and wheat germ agglutinin binding to purify osteoblastic cells from the 5-fluorouracil treated femoral BM of young adult mouse (Van Vlasselaer et al., 1994). Two mAbs (OB 7.3 and SB-5) have been raised that label the surface of chick osteocytes specifically and OB 7.3-coupled magnetic beads have been used to isolate relatively pure osteocytes from chick mixed bone cell populations (Van der Plas and Nijweide, 1992). Several antibodies are available that recognize early as well as late stage osteoblastic cells, e.g. RCC455.4, which we recently isolated (Turksen et al., 1992) and found by expression cloning to recognize galectin 3 (Aubin et al., 1996). Immunopanning of human BM cells with osteocalcin and osteonectin enriches for osteoblastic cells (Long et al., 1995). Antibodies against AP have been raised by several labs (in our laboratory, RBM 211.13 was raised against RBM stroma (Turksen et
al., 1992)); these label preosteoblastic cells as well as osteoblasts. In the rat calvarial system, AP expression has been used successfully to fractionate osteoblastic populations into AP-positive and -negative populations (Turksen and Aubin, 1991). Most of the osteoprogenitors giving rise to osteoblasts making bone in vitro in the absence of added glucocorticoids (dex) reside in the AP-positive pool, while osteoprogenitors present in the AP-negative pool require dex to differentiate. AP expression has also been used for flow cytometric sorting in female RBM stromal cell cultures, but production of a mineralized matrix after sorting was only described in cells which were AP negative at the time of sorting (Rickard et al., 1994). There are few reports that describe antibodies recognizing cells earlier than the preosteoblast. Haynesworth et al. (1992) immunized with human marrow stromal cells to isolate antibodies that react on a subset of marrow stromal cells and a variety of tissues in vivo, but do not label osteoblasts or osteocytes (Haynesworth et al., 1992). The authors concluded that the antibodies may recognize molecules present on early progenitors or stem cells and that the epitope(s) is lost during osteogenic differentiation, but conclusive support for this awaits further characterization (Haynesworth et al., 1992). STRO-1 recognizes mesenchymal progenitors in human marrow stromal cells with the ability to form cells with the phenotype of fibroblasts, adipocytes, smooth muscle cells, and osteoblasts (Simmons and Torok-Storb, 1991; Gronthos et al., 1994). Recently, HOP-26 was described as an antibody strongly reactive with cells in marrow stromal colonies at early stages of differentiation, before the induction of AP, and HOP-26 does not bind to mature osteoblasts; immunopanning with HOP-26 selected the marrow stromal fibroblastic colony-forming units (CFU-F) (Joyner et al., 1997). Bruder and colleagues (Bruder et al., 1997) have also recently reported an antibody which they believe recognizes human mesenchymal stem cells. As yet, similar antibodies to early progenitors have not been reported for the RBM stromal system.
1.6 Cytokines and Growth Factors in the Stromal Environment

The rich cytokine milieu resulting from production by both hematopoietic and stromal cells creates a complex environment in which both immature osteoprogenitors and mature osteoblasts reside. For example, G-CSF (Felix et al., 1988; Greenfield et al., 1993; Taichman and Emerson, 1994), GM-CSF (Felix et al., 1988; Horowitz et al., 1989; Horowitz et al., 1989; Weir et al., 1989; Felix et al., 1991; Benayahu et al., 1992; Greenfield et al., 1993), M-CSF (Sato et al., 1986; Shiina-Ishimi et al., 1986; Elford et al., 1987; Sato et al., 1987; Felix et al., 1989; Greenfield et al., 1993; Weir et al., 1993; Lacey et al., 1994), IL-1α (Greenfield et al., 1993), IL1β (Birch et al., 1993; Dodds et al., 1994; Bilbe et al., 1996; Shadmand and Aubin, 1997), IL-3 (Felix et al., 1988; Birch et al., 1993), IL-5 (Greenfield et al., 1993), IL-6 (Ishimi et al., 1990; Birch et al., 1993; Greenfield et al., 1993; Dodds et al., 1994; Lacey et al., 1994; Bilbe et al., 1996; Malaval et al., 1997; Xiong et al., 1997), IL-7 (Greenfield et al., 1993), IL-8 (Birch et al., 1993; Chaudhary and Avioli, 1994; Bilbe et al., 1996), IL-11 (Bilbe et al., 1996), LIF (Greenfield et al., 1993; Bilbe et al., 1996; Malaval et al., 1997), ONC-M (Bilbe et al., 1996), PDGF-α (Graves et al., 1989; Zhang et al., 1991; Bilbe et al., 1996), PDGF-β (Bilbe et al., 1996; Canalis and Rydziel, 1996), SCF (Bilbe et al., 1996), TGF-β (Birch et al., 1993; Greenfield et al., 1993; Dodds et al., 1994; Bilbe et al., 1996), TNF-α (Birch et al., 1993; Greenfield et al., 1993; Bilbe et al., 1996), and TNF-β (Bilbe et al., 1996) are produced by osteoblastic cells. Glucocorticoids are known important regulators of cytokine and growth factor production in many cell types, and this laboratory has been particularly interested in glucocorticoid regulation of osteoprogenitor differentiation in both calvarial cell cultures and in the RBM stromal populations. In RBM stromal cultures, there is little bone formation in the absence of dex. Work reported in this thesis will address whether the effect of dex is direct on osteoprogenitor cells or indirect through its ability to alter the numbers and kinds of other lineages present which would concomitantly alter the cytokine and growth factor milieu of the stromal cultures. In
particular, the ability of dex to stimulate the maturation of monocyte lineage cells into macrophages (see Chapter 2 and (Herbertson and Aubin, 1995)) would be expected to alter the levels and kinds of cytokines and factors produced by cells of this lineage, including PDGF, whose effects on osteoprogenitor differentiation I have investigated in detail (Chapter 4).

1.7 Platelet-Derived Growth Factor (PDGF)

PDGF - General Notes
PDGF is well-known as a mitogen for mesenchymal cells and appears to have broad roles in tissue development. PDGF exists as 30 kDa disulphide bonded homo- or hetero-dimers of A and B chains which interact with the two PDGF receptors, α and β. PDGF-B, the normal counterpart of the oncogene v-sis of the simian sarcoma virus (Westermark and Heldin, 1991), has been shown to have stimulatory effects on primitive pluripotential hemopoietic cells in mouse BM (Yan et al., 1993) and overexpression of PDGF-B in murine hemopoietic cells induces a lethal myeloproliferative syndrome in vivo with anemia, neutrophilia and monocytosis (Yan et al., 1994). PDGF and its receptors show appositional expression in epithelial/stromal tissues, and appear to be involved in stromal-mesenchymal interactions during normal embryonic development and in basal cell carcinomas (Ponten et al., 1994). Measurements of transcript levels during mouse development have shown that PDGF A chain and PDGF α-receptor are expressed early, while PDGF B chain and PDGF β-receptor appear to become important later in development (Bowen-Pope et al., 1991). In normal embryos, PDGF α-receptor is expressed in both mesodermal and neural crest derived mesenchyme (Schatteman et al., 1992). The PDGF α-receptor gene is deleted in the Patch mutant mouse (Bowen-Pope et al., 1991); in homozygous mutants many mesodermal derivatives are poorly developed and the embryos do not survive until birth (Bowen-Pope et
al., 1991). Specifically in terms of skeletal effects, homozygous embryos demonstrate severe vertebral malformations, spina bifida and cleft face, and distortions of other bones formed by endochondral ossification (Schatteman et al., 1992). The Patch heterozygotes express half the wild-type level of PDGF α-receptor transcripts and protein and survive with minimal defects: an increase in the width of the prefrontal bone and absence of functional melanocytes (Bowen-Pope et al., 1991). The Patch mutant mice suggest that PDGF is very important in mesodermal and skeletal development.

**PDGF in Non-Osteogenic Stroma**

Fibroblasts and macrophages are sources of PDGF (Bowen-Pope et al., 1991) and we have previously reported that both cell types are present in RBM stromal cells under conditions which promote bone formation (Herbertson and Aubin, 1995). PDGF appears to be stored in the bone matrix, and the trapping or sequestering of PDGF could affect its biological action through complex modes of release and presentation to responding cells (Hauschka et al., 1986).

**PDGF in Osteoblasts**

Normal human osteoblastic cells produce PDGF-A (Graves et al., 1989; Zhang et al., 1991), PDGF α- and β-receptors (Zhang et al., 1991). PDGF B is reportedly not made by human osteoblastic cells (Graves et al., 1989; Zhang et al., 1991; Bilbe et al., 1996), although there is a recent report of its production by rat osteoblasts (Canalis and Rydziel, 1996) and mRNA for PDGF B have been detected in several human osteoblastic cell lines (MG-63 cell line, SaOs-2, TE-85) (Bilbe et al., 1996). The effect of PDGF on osteogenic cells in vitro has been investigated by several groups. PDGF increases proliferation (fetal rat calvaria organ cultures (Canalis and Lian, 1988; Pfeilschifter et al., 1990), fetal rat calvaria cell cultures (Centrella et al., 1989; Centrella et al., 1991; Pfeilschifter et al., 1992), human osteoblastic cells (Graves et al., 1989; Gilardetti et al., 1991; Zhang et al., 1991; Abdennagy et al., 1992),
mouse trabecular explant cells (Abdennagy et al., 1992) and the murine osteoblastic cell line MC3T3-E1 (Davidai et al., 1992)), consistent with PDGF's well known activity as a mitogen for mesenchymal cells including fibroblasts, glial cells, and smooth muscle cells. However, the evidence in terms of osteoblast proliferation is not conclusive, as most experiments with nontransformed cells involve possibly mixed cultures, with fibroblasts and other stromal cells present which are also known to respond mitogenically to PDGF (Hirata et al., 1985), and it has been shown that in fetal rat calvarial tissue culture, PDGF selectively stimulated fibroblast replication and function (Hock and Canalis, 1994).

**Effects of PDGF on Osteoblastic Cells In Vitro**

The reported effects of PDGF on osteoblast differentiation have been controversial (no effect - (Canalis and Lian, 1988; Cassiede et al., 1996); positive effect - (Centrella et al., 1989; Pfeilschifter et al., 1990); negative effect - (Centrella et al., 1991; Pfeilschifter et al., 1992; Hock and Canalis, 1994)). With respect to the effect of PDGF on differentiation, there are several possible sources of variation, including the PDGF isoform used. Although the two PDGF receptors are structurally and functionally related, there are important differences between them in ligand binding: the α-receptor binds all three PDGF isoforms (PDGF-AA, -AB, -BB) with similarly high affinities, but the β-receptor binds only PDGF-BB with high affinity and binds PDGF-AB with a 10-fold lower affinity (Hart, 1990; Grotendorst et al., 1991). The proportions of PDGF receptor types can vary between different cell types (Hart, 1990). In fetal rat calvaria cell populations, osteoblasts express primarily αβ receptor complexes, with 30% ββ receptor and 10% αα receptor complexes (Pfeilschifter et al., 1992). There also appear to be important differences between PDGF receptor types in responses elicited (Pfeilschifter et al., 1992) and the two receptors initiate traverse of the cell cycle by distinct mechanisms (Coats et al., 1992). In human fibroblasts the PDGF β-receptor, and not
Table 1.1 The Effects of PDGF on Proliferation and Differentiation in Osteoblastic Cell Cultures

<table>
<thead>
<tr>
<th>Study</th>
<th>Culture</th>
<th>Prolif'n</th>
<th>Differentiation (#)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canalis and Lian (1988)</td>
<td>FRC* organ culture</td>
<td>+</td>
<td>no effect (collagen/osteocalcin)</td>
</tr>
<tr>
<td>Graves et al. (1989)</td>
<td>adult human cell cultures</td>
<td>+</td>
<td>not determined</td>
</tr>
<tr>
<td>Centrella et al. (1989)</td>
<td>FRC cell cultures</td>
<td>+</td>
<td>+ (collagen/total protein)</td>
</tr>
<tr>
<td>Pfeilschifter et al. (1990)</td>
<td>FRC organ cultures</td>
<td>+</td>
<td>+ (collagen)</td>
</tr>
<tr>
<td>Zhang et al. (1991)</td>
<td>explant human bone cells</td>
<td>+</td>
<td>not determined</td>
</tr>
<tr>
<td>Centralla et al. (1991)</td>
<td>FRC cell culture</td>
<td>+</td>
<td>- (AP)</td>
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<tr>
<td>Gilardetti et al. (1991)</td>
<td>adult human cell culture</td>
<td>+</td>
<td>not determined</td>
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<td>Pfeilschifter et al. (1992)</td>
<td>FRC cell culture</td>
<td>+</td>
<td>+ (collagen)</td>
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<td></td>
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<td>Hock and Canalis (1994)</td>
<td>FRC organ cultures</td>
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<td></td>
<td></td>
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<td>- (bone matrix formation)</td>
</tr>
<tr>
<td>Cassiede et al. (1996)</td>
<td>RBM stromal cell cultures</td>
<td>+</td>
<td>- (AP)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>no effect (osteogenesis)</td>
</tr>
</tbody>
</table>

*FRC = fetal rat calvaria

# = markers used as an indicator of bone differentiation are included in parentheses
the α-receptor, mediated induction of actin reorganization and membrane ruffling (Westermark and Heldin, 1991). U-2 OS cells migrated in the presence of PDGF AB and BB dimers but not in the presence of PDGF AA dimers (Allam et al., 1992), suggesting the chemotactic effect is mediated through the β-receptor. Thus the use of a particular isoform, or a mixture of isoforms, may yield different results depending on the levels and kinds of PDGF receptors a particular cell type expresses.

The fact that different culture systems (e.g., fetal versus adult, tissue versus cell, clonal versus mixed population) and different culture conditions (e.g., serum supplementation or not) are being used to monitor osteoblast responsiveness to PDGF exacerbates the difficulty. The maturational status or differentiation of progenitor populations can also lead to changes in receptor levels: although PDGF α-receptor appears to be present throughout osteoblast development, the levels appear to increase with osteoblast differentiation (Liu and Aubin, 1996). The duration of exposure to PDGF may also be important; most investigations have used short exposure times (24 hr.) (Canalis and Lian, 1988; Centrell et al., 1989; Zhang et al., 1991) or 48 hr. (Pfeilschifter et al., 1990) to 72 hr. (Hock and Canalis, 1994). Given earlier data with other factors such as EGF (Antosz et al., 1987), which show biphasic effects depending upon the time and duration of exposure, longer exposures may have significantly different effects from shorter exposures. The timing of both the pulse and the phenotypic assessments are also important, since recently it was reported that assessments of proliferation and AP activity of marrow stromal cells immediately after exposure to PDGF were different from assessments done later (Cassiede et al., 1996). Another possible reason for reported differences is that different markers of osteoblast development have been used in different studies. For example, total protein synthesis, collagen synthesis, AP and osteocalcin production have all been used as differentiation markers. While PDGF has been shown to increase collagen synthesis (Centrella et al., 1989; Pfeilschifter et al., 1990), the collagen may be incorporated into a fibrous tissue matrix instead of bone matrix.
Experiments which use the more specific bone markers such as AP (although not unique to the osteoblast lineage) or the highly specific osteocalcin suggest that PDGF inhibits bone formation in vitro (Centrella et al., 1992; Pfeilschifter et al., 1992), consistent with reports that in calvaria tissue culture, PDGF inhibits bone matrix formation (Hock and Canalis, 1994). This may be in keeping with the effect of PDGF on smooth muscle cells: PDGF-BB increases proliferation while inhibiting expression of the differentiated smooth muscle cell phenotype (Holycross et al., 1992).

**Effects of PDGF on Osteoblastic Cells In Vivo**

Similarly to the in vitro studies, there are discrepancies in terms of the effect of PDGF on bone in vivo. PDGF (A and B) is expressed at sites of normal human fracture repair, suggesting a role in regulation of this process (Andrew et al., 1995). Local administration of PDGF-BB at the time of osteotomy in rabbits had a stimulatory effect on bone fracture healing in terms of increased callus density, increased mechanical bone strength, and osteogenic differentiation (Nash et al., 1994). In contrast, local administration of PDGF-BB in rat craniotomy defects was found to inhibit osteogenin induced bone regeneration and stimulated a soft tissue repair wound phenotype (Marden et al., 1993). Systemic administration of PDGF-BB to adult female rats significantly increased osteoblast number, bone density and strength, although premature closure of the growth plate, decreased body fat, and extraskeletal collagen deposition were also noted (Mitlak et al., 1996). The differing results in the in vivo studies may also be due to the different systems and methods of administration of PDGF. PDGF is not normally found in plasma and plasma appears to contain PDGF binding protein(s) that would serve to inactivate PDGF released into plasma (Bowen-Pope et al., 1984). Intravenously injected PDGF is rapidly cleared from the plasma in baboons (t½ = 2 min. (Bowen-Pope et al., 1984)) and in rodents (90% removed after 1 hr. (Cohen et al., 1990). These results suggest that release of PDGF at sites of vascular injury would greatly increase the local concentration of PDGF but PDGF not localized to the
site of injury would be rapidly cleared from the circulation (Bowen-Pope et al., 1984). Thus, systemic administration of PDGF likely results in only short pulse exposures. In summary, while PDGF is thought to have critical roles in proliferation during development and during fracture healing, its role in osteoprogenitor differentiation and bone formation is still poorly understood.
1.8 Thesis Objectives

The objective of this thesis was to better define and characterize the heterogeneous cellular environment of the rat bone marrow (RBM) stromal cell osteogenic system. To this end, I first used histochemical and immunohistochemical methods to identify and quantify the main subpopulations present in RBM stroma and determine their time course of development under conditions supporting osteogenesis (Chapter 2). I then applied methods to separate and enrich the osteogenic population from the other stromal populations (Chapter 3). Finally, I investigated the effects of PDGF on RBM stromal osteogenesis using both the mixed populations and the enriched populations (Chapter 4).
CHAPTER 2

Dexamethasone Alters the Subpopulation Make-Up of Rat Bone

Marrow Stromal Cell Cultures
2.1 Introduction

The stromal system of BM refers to the connective tissue elements which provide structural and functional support for hemopoiesis. Osteogenic cells are known to be present in the BM stroma of various animal models (Maniatopoulos et al., 1988; Benayahu et al., 1989; Friedenstein, 1990; Leboy et al., 1991) including human marrow (Haynesworth et al., 1992), and production of a bone-like mineralized tissue from marrow cells has been demonstrated both in vivo, i.e., in diffusion chambers loaded with BM cells (Friedenstein, 1968), and in vitro, where bone-like tissue is synthesized by marrow stromal cells cultured in medium containing ascorbic acid, β-glycerophosphate and the glucocorticoid dex (Maniatopoulos et al., 1988; Kasugai et al., 1991; Satomura and Nagayama, 1991; Malaval et al., 1992). However, in addition to osteogenic cells, BM stroma contains other cell types: those widely acknowledged include fibroblasts, adipocytes, and endothelial cells (Dexter, 1982; McIntyre and Bjornson, 1986; Owen, 1988; Hasthorpe et al., 1990; Wang and Wolf, 1990; Benayahu et al., 1991; Bennett et al., 1991). Other cell types identified as components of BM stroma include smooth muscle cells (Peled et al., 1991; Simmons and Torok-Storb, 1991; Galmiche et al., 1993), reticular (Dexter, 1982; Benayahu et al., 1991) and reticulo-fibroblastoid cells (Ross et al., 1991).

Hemopoietic cells exist in intimate association with stromal cells in BM in vivo and in vitro. Long term BM cultures have been used to unravel the differentiation process of many white blood cell lineages, as well as the role of stromal lineages in this process (Dexter, 1982). When stromal cells are cultured, the majority of well-differentiated hemopoietic cells are thought to be removed by centrifugation or, as in our system, by adherence techniques. However, hemopoietic stem cells are present at a frequency of 1 in 25,000 adherent marrow cells (Kiefer et al., 1991), and culturing the adherent BM fraction results in the culturing of early hemopoietic cells whose development is guided by the existing culture conditions. In
mice, macrophages are frequently seen in cultures of BM stromal cells (Dexter, 1982) and isolated macrophage colonies can be obtained at low plating density (Wang and Wolf, 1990). Dendritic cells, which belong to the myeloid lineage, have also been described in stromal cultures (Giesler et al., 1991; Inaba et al., 1992).

Although there are some data describing the different cell types in RBM stroma, there is little quantitative information on the subpopulation make-up. One study of primary RBM cultures after 10 d. growth in vitro reported largely fibroblast-like (85%) colonies, and by immunoperoxidase staining 10.3% macrophages-dendritic and granulocytic cells, and 2.5% endothelial cells (Simmons et al., 1991). Since it is known that the culture conditions have a significant effect on what lineages proliferate and differentiate, the subpopulations present need to be determined for each system. There are scant data on the subpopulation make-up when stromal cells are grown under conditions stimulating bone formation. When RBM stromal cells were cultured under such conditions (Maniatisopolous et al., 1988), fibroblast colony forming units (CFU-F) were found to comprise about 1/100th of the adherent cell fraction, while osteoprogenitor cells giving rise to bone nodules comprise about 1/300th of the same plated population in the presence of dex (Aubin et al., 1990). Immunocytochemistry, in combination with Northern analysis and morphological description, confirmed that only a portion of the cells contribute to osteogenesis (Malaval et al., 1994). Neither CFU-F nor osteoprogenitor expression followed a linear relationship in limiting dilution analysis suggesting cooperativity of cell types within the population and a multi-target phenomenon (Aubin et al., 1990). In support of interacting cell types, when a large constant number of nonadherent (hemopoietic) BM cells was added back to adherent stromal cells, osteoprogenitor cells were measured at a frequency of 1/100th adherent cells and their expression followed a linear, single-hit relationship. Endothelial cells or endothelial cell-conditioned medium also increased expression of osteoprogenitors giving rise to bone nodules (Aubin et al., 1990). These data and others (Hughes and McCulloch,
1991; Ogiso et al., 1991; Zhang et al., 1991) indicate that the expression of osteogenesis by BM osteoprogenitor cells may be under the regulation of other cells in the BM, which can exert either stimulatory or inhibitory activities. As a step toward understanding further the cell-cell interactions affecting bone formation in the RBM stromal system, we have analyzed quantitatively the subpopulations over a temporal sequence during which osteoprogenitors differentiate and bone nodules form.

2.2 Materials and Methods

Cell culture and plating
BM stromal cells from the femora of young 40 - 43-day-old, 110 - 120 gram, male Wistar rats were cultured essentially as described (Maniatopoulos et al., 1988). The rats were sacrificed by cervical dislocation, and the femora were dissected under aseptic conditions and placed in medium (α-modified essential medium, α-MEM) containing antibiotics (1000 ug/ml penicillin G (Sigma Chemical Co., St. Louis, MO), 500 ug/ml gentamycin sulfate (Sigma), and 3.0 ug/ml Fungizone (Flow Laboratories, McLean, VA)) (10xAB). The adherent connective tissue and muscle were removed from the exterior of the femora, the femora were placed in fresh antibiotic medium as above, and their ends were cut off with a scalpel. Each femur was flushed with α-MEM until the bone appeared blanched (about six or eight times). This suspension was passed through a syringe several times to produce a largely single cell suspension; cells recovered from two femora were added to a T-75 tissue culture flask (Falcon, Becton Dickinson, Oxnard, CA) and incubated for one week in a 37°C humidified 95% air/5% CO₂ incubator. Growth medium, consisting of α-MEM containing 10% fetal calf serum, antibiotics (100 ug/ml pen G, 50 ug/ml gentamycin, and 0.3 ug/ml Fungizone), 50 ug/ml ascorbic acid, was changed every 2 - 3 d. After 7 d., each T-75 cell culture flask was washed with 15 ml warm PBS and adherent cells were recovered with a
mixture of 10 ml of 0.25% trypsin (w/v in citrate saline) and 5 ml of collagenase (Rao et al., 1977). Recovered cells were passed through a syringe with a 22 gauge needle to ensure a single-cell suspension. Cells were counted electronically on a Coulter Counter then plated at densities between $8 \times 10^3$ and $5 \times 10^4$ cells per 35mm dish (Falcon), or at a range of these densities to obtain a balance between colony isolation and adequate colony numbers for quantitation (see Results section). For flow cytometry, cells were plated in 100 mm dishes (Falcon). Cells were cultured in α-MEM supplemented as above, and where indicated, $10^{-8}$ M dex (Sigma) for various periods of time from 7 - 35 d. To promote mineralization, 10 mM Na-β-glycerophosphate (Sigma) was added either continuously or for the 2 d. of culture prior to fixation. Cultures were used to determine total cell number, stained, or prepared for flow cytometry as below.

Cell Counts

Cells were recovered from a minimum of triplicate dishes by trypsinizing as above and counted on a Coulter Counter.

Staining

AP/von Kossa/toluidine blue

The histochemical stain for AP is a modification of Pearse's (1960) method (Drury and Wallington, 1967). Cells were rinsed once with cold PBS and fixed in 10% cold neutral buffered formalin for 15 min., rinsed with distilled water, and left in distilled water for 15 min. Fresh substrate (10 mg Naphthol AS MX-PO4 (Sigma) dissolved in 400 ul N,N-dimethylformamide, added to 50 ml distilled water and 50 ml Tris-HCl (0.2 M, pH 8.3) and 60 mg Red Violet LB salt (Sigma)), filtered through Whatman's No. 1 filter immediately before use, was added and incubated for 45 min at 20°C. The dishes were then rinsed in distilled water, drained and stained with 2.5% silver nitrate for 30 min. at 20°C (von Kossa stain). The dishes were again rinsed in distilled water and drained, toluidine blue was
applied for 2 seconds and the dishes were then rinsed 3 times with tap water. The dishes were finally air dried.

\[ \alpha \text{NBE} \]

The procedure from Sigma Diagnostics Kit 181 (St. Louis, USA) for \( \alpha \text{NBE} \) was followed, except that the counterstaining was omitted. The kit describes the staining patterns for monocytes and T lymphocytes, but macrophage staining with this stain has been described in several studies (Giesler et al., 1991; Inoue et al., 1991; Kanemoto et al., 1991).

\[ \text{Sudan IV} \]

Sudan IV staining method is modified from Clark (Clark, 1981). Medium was suctioned from the culture dishes and cells were fixed in 10% neutral buffered formalin for 1 hr. After rinsing with distilled water, followed by rinsing twice rapidly with 70% alcohol, Sudan IV solution (0.1 gm Sudan IV, 50 ml acetone, 50 ml alcohol) was added for 5 - 10 minutes. Differentiation of the stain was done with 70% alcohol for 2 - 5 seconds, then the dishes were rinsed with water and allowed to air dry.

\[ \text{Horseradish Peroxidase Conjugated Antibody Staining} \]

Medium was suctioned from the culture dishes, cells were fixed in 3.7% formalin in TBS (10 mM TRIS pH 7.5, 150 mM NaCl) for 5 minutes, followed by -20° methanol for 5 minutes if permeabilization was needed. Endogenous peroxidase activity was blocked with 3% \( \text{H}_2\text{O}_2 \) in TBS. The first antibodies, either RBM 211.13, anti-rat AP (Turksen et al., 1992) (x1000 dilution) or ED2 anti-rat macrophage (Dijkstra et al., 1985) (Serotec Ltd., Toronto, Canada, diluted x500) were diluted in TBS containing 3% BSA prior to addition, and cell cultures incubated at 37° for 45 - 60 minutes. After washing, horseradish peroxidase-conjugated (HPR) GAM (diluted x300) was added and incubation continued at 37°C for 30 - 45 minutes. HPR Colour Development Reagent (0.3 g. BioRad CDR in 100
ml of -20° methanol plus 60 µl H₂O₂ (30%) in 100 ml TBS, mixed in equal proportions immediately before using) was added at 20° for one hr., cells were washed in H₂O and allowed to air dry.

**Quantitation**

Quantitation of colony types involved identification of AP positive colonies by staining as above, bone nodules by morphology and von Kossa staining, macrophages by αNBE and ED2 staining, and adipocytes by morphology and Sudan IV staining. For quantitation, only foci containing >10 cells (approximately three population doublings) were classified as colonies; both colony counts and grid point counts (percentage of grid cross points covered by colonies) for proportion of the culture dish covered were done. In some experiments, colonies were graded according to their size (10 - 25 cell foci, 25 - 100 cells, or > 100 cells). For statistical analysis, the Student t test (unpaired, double-sided) was used to determine levels of significance.

**Flow cytometry**

Cells were recovered from dishes by a mixture of trypsin-collagenase as above, passed through a syringe with a 22 gauge needle, and centrifuged. The cells were resuspended in fresh growth medium as above and incubated at 37°C for 30 - 60 minutes to allow the cells to recover from trypsinization. Cells were centrifuged then resuspended in sterile primary mouse anti-rat antibodies (mouse myeloma Ig's (Organon Teknika Co., West Chester, PA) as a control, RBM 211.13 for AP(Turksen and Aubin, 1991), ED2 (Serotec) for macrophages (Dijkstra et al., 1985), MRC OX-1 (Cedarlane Laboratories Ltd., Canada) for leukocyte common antigen (Sunderland et al., 1979) or MRC OX-22 (Serotec) for leukocyte common antigen restricted to T and B lymphocytes (Woollett et al., 1985) diluted in 3% BSA in PBS, and incubated at 37°C for 30 minutes. After incubation, cells were washed, incubated with FITC-conjugated sheep anti-mouse antibodies (Serotec) for 30 minutes at
37°C, and then washed with PBS. Immediately before analysis, cells were filtered through a fine mesh to remove any cell clumps. Propidium iodide was added to determine cell vitality. Samples were run on a Coulter EPICS Profile Analyzer equipped with an Argon laser operated at 488 nm for fluorescence excitation. Fluorescence was detected with a 525 nm band pass filter (+/- 10 nm) and samples gated for size and low propidium iodide staining.

2.3 Results

RBM stromal cells cultured for 21 d. in the presence of 10% FCS, ascorbic acid, β-glycerophosphate, and dex, produced mineralized bone nodules similar to those previously characterized as bone-like (Maniatopoulos et al., 1988; McCulloch et al., 1991). When marrow stromal cells were cultured as above but without dex, bone nodules were also seen, but at a much lower frequency. The difference in bone nodule formation in cultures with and without dex is apparent macroscopically (see Figure 2.1, A and B). Microscopic assessment of bone nodule numbers showed significantly greater numbers in the cultures with dex, compared to those without, in six out of six separate experiments, three of which are shown in Table 2.1. An increase in the numbers of bone nodules in cultures with dex was accompanied by a significant decrease in total cell number in these cultures (data not shown), consistent with previous studies (Maniatopoulos et al., 1988). Cells associated with early bone nodules exhibited typical polygonal osteoblast morphology and stained positively for AP (see Figure 2.5A). Bone nodules formed in these cultures over several days; the number of bone nodules formed plateaued by d. 21 (Figure 2.2) but mineralization detected by von Kossa staining continued to increase up to at least 35 d. when β-glycerophosphate was present continuously. Because of the variability of the frequencies of different cell types within each BM isolation, each separate experiment used a minimum of three different plating densities in order to obtain bone nodules in numbers satisfactory for both counting.
and statistics (i.e., 10-150 nodules per 35 mm dish). In Table 2.1, the plating density for each separate experiment is noted. Both time at which new nodule formation ceased and especially the onset of mineralization were plating cell density-dependent: both occurred earlier when higher densities of bone nodules were present (data not shown).

In cultures in which bone nodules formed, many other cell morphologies and colony types were also evident. Using the same cell cultures as those in which bone nodules were analyzed and the same temporal sequence, we identified and quantitated these other marrow stromal populations with a variety of histochemical stains. In six out of six experiments, a large percentage of the colonies present were positive for AP, with significantly higher numbers of AP positive colonies in cultures grown with dex (three of which are shown in Table 2.1). Similarly to the time course of bone nodule formation, the percentage of a culture dish covered by AP positive colonies (as determined by grid point count) plateaued after 21 d. However, only a proportion of the AP positive colonies were coincident with and corresponded to bone nodules (Figure 2.3A, 2.5A). A micrograph of an AP positive colony with a nonbone appearance is shown in Figure 2.5B. Although the total number of AP positive colonies appeared to decrease after d. 14 (Figure 2.3A), this is likely due to an increase in numbers of large AP positive colonies which merge with and become indistinguishable from the smaller colonies (see Figure 2.3B).

Pronounced differences in αNBE positive (monocyte/macrophage) colonies (Figure 2.5D) in cultures with and without dex was also apparent. The number of αNBE positive colonies was significantly higher in dex-containing cultures in six out of six experiments, three of which are shown in Table 2.1. In contrast to the time course of formation of AP positive colonies and bone nodules, all sizes and total number of αNBE positive (monocyte/macrophage) colonies continued to increase up to at least d. 28 of culture, the last
time point analyzed (see Figure 2.4). There was no apparent consistently close physical association of bone nodules and αNBE positive colonies (Figure 2.5D).

Fat colonies (Figure 2.5C) were present at very low frequency under our culture conditions, and dex significantly increased the numbers of fat colonies in only one out of six of these experiments, and only slightly in that particular experiment (see Table 2.1).

Immunolabelling was used to identify more specifically the hemopoietic colonies. ED2 (macrophage) immunohistochemically stained colonies (Figure 2.6B) were of similar cell size and morphology as those strongly positive with αNBE. However, ED2 positive colonies were present at similar or higher frequencies to strongly positive αNBE colonies, probably reflecting the increased sensitivity of the immunohistochemical stain over the histochemical stain. Quantitation of ED2 positive colonies indicated that macrophage number increased in cultures grown with dex compared with those without dex in four out of four separate experiments, three of which are shown in Table 2.1.

We also used flow cytometry to further quantify the stromal cell make-up under these growth conditions. The stromal population exhibited a broad distribution of cell sizes consistent with the many subpopulations present. The number of cells positively labeled were compared with control cells incubated with nonspecific mouse myeloma Ig's. Similar to the results from the colony counts, there were more AP positive cells and more macrophages in the cultures with dex compared with the cultures without dex. For example, in the experiment shown in Figure 2.7, in the cultures with dex compared to those without, 29.7% versus 12.5%, respectively, of total cells were labeled positively for AP, and 8.3% versus 2.5%, respectively, of total cells were positive with ED2 antibody. Flow cytometry was more sensitive than immunohistochemical staining in that it identified relatively more macrophages in the cultures without dex than were apparent by colony counting (the results
from Figure 2.7 are from the same experiment shown as experiment 3 in Table 2.1). Consistent with the cells seen in culture dishes (compare Figure 2.6C to 2.6A), flow cytometry identified more hemopoietic cells (MRC OX-1 positive) in the cultures without dex compared to those with dex (Figure 2.7D versus 2.7H). While the majority of the hemopoietic cells in the cultures with dex are macrophages (8.3% macrophages out of 11.5% of cells identified as hemopoietic cells), a much smaller proportion of the hemopoietic cells in the cultures without dex are macrophages (2.5% macrophages out of 36.9% hemopoietic cells). The bulk of the hemopoietic cells in the cultures without dex are as yet unidentified, but do not appear to be lymphocytes as determined by lack of labeling with MRC OX-22 (data not shown). They do not appear to be of the granulocytic lineage as they lack the typical morphology and previous histochemical staining trials with naphthol AS-D chloroacetate esterase (Sigma) and myeloperoxidase (Sigma) for neutrophils and their precursors were also negative (data not shown).
Table 2.1 Quantitation of colony types identified in RBM stromal cell cultures grown for 21 d. in vitro with ascorbic acid, β-glycerophosphate, and with or without dex (10⁻⁸ M)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Plating density (35mm dish)</th>
<th>(+) or (-) dex</th>
<th># bone nodules</th>
<th>% covered with AP+ colonies</th>
<th># fat colonies</th>
<th># αNBE+ colonies</th>
<th># ED2+ colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 x 10⁴</td>
<td>+</td>
<td>34 ± 4</td>
<td>89.1 ± 2.1</td>
<td>4 ± 2</td>
<td>73 ± 10</td>
<td>91 ± 10</td>
</tr>
<tr>
<td></td>
<td>5 x 10⁴</td>
<td>-</td>
<td>0 ± 0</td>
<td>82.9 ± 1.1</td>
<td>1 ± 1</td>
<td>3 ± 1</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>2</td>
<td>5 x 10⁴</td>
<td>+</td>
<td>112 ± 7</td>
<td>98.4 ± 0.2</td>
<td>0 ± 0</td>
<td>119 ± 5</td>
<td>259 ± 11</td>
</tr>
<tr>
<td></td>
<td>5 x 10⁴</td>
<td>-</td>
<td>1 ± 1</td>
<td>95.8 ± 0.2</td>
<td>1 ± 1</td>
<td>7 ± 2</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>3</td>
<td>2 x 10⁴</td>
<td>+</td>
<td>11 ± 2</td>
<td>92.4 ± 0.7</td>
<td>0 ± 0</td>
<td>52 ± 5</td>
<td>81 ± 4</td>
</tr>
<tr>
<td></td>
<td>2 x 10⁴</td>
<td>-</td>
<td>0 ± 0</td>
<td>87.4 ± 1.2</td>
<td>0 ± 0</td>
<td>11 ± 4</td>
<td>0.4 ± 0.2</td>
</tr>
</tbody>
</table>

Experiments with + dex > - dex: 3/3*** 3/3** 1/3* 3/3*** 3/3***

T-Test of (+) dex versus (-) dex: * p ≤ .05; ** p ≤ .01; *** p ≤ .001
Figure 2.1 Overviews of 35 mm culture dishes grown for 21 d. in medium containing ascorbic acid and β-glycerophosphate. Dishes were stained with AP/von Kossa/toluidine blue; cells cultured with dex (A) or cultured without dex (B). Black von Kossa positive nodules are apparent in A, while AP positive colonies appear gray in both (magnification 1.6x).
Figure 2.2 Time course of formation of bone nodules in RBM stromal cells cultured with $10^{-8}$ M dex. Bone nodules first appeared during the second week of culture and their number appeared to plateau during the third week of culture; the proportion of nodules mineralized appeared to increase with time in culture. In this experiment, $\beta$-glycerophosphate was present continuously. ■ mineralized nodules; □ total bone nodules
Figure 2.3 (A) Comparison of the number of AP positive colonies ( ), the area of the culture dish they cover ( ), and the number of bone nodules ( ) in RBM stromal cells cultured for various periods of time with dex. Only a proportion of the AP positive colonies are coincident with bone nodules. (B) Size distribution of AP positive colonies seen in (A) with time. While the number of small colonies and foci decrease with time, the number of large colonies increase with time. Colony size distributions: 10 - 25 cells; 25 - 100 cells; > 100 cells
Figure 2.4 All size classes of strongly positive αNBE colonies in RBM stromal cells, cultured with dex, continued to increase at least up to d. 28, the latest point analyzed. Colony size: 10 - 25 cells; 25 - 100 cells; > 100 cells. Total colonies:
Figure 2.5 Photomicrographs of the different cell types present in RBM stromal cells cultured for 21 d. with dex. (A) AP/von Kossa/toluidine blue staining of a bone nodule where * marks mineralized von Kossa positive nodule and AP positive (appear gray in this black and white photomicrograph) cells with typical polygonal morphology can be seen at the periphery of the mineralized nodule (magnification x100). (B) AP positive cells (appear gray) (magnification x100). (C) Sudan IV positive (fat) cells showing a colony with two foci; stained lipid droplets appear black in this black and white photomicrograph (magnification x30). Inset shows an adipocyte with typical morphology (magnification x300). (D) αNBE positive (monocyte/macrophage) cells (appear black) not associated with a bone nodule (magnification x170).
Figure 2.6 Photomicrographs of immunohistochemical staining of RBM stromal cells grown for 21 d. (A) Control cells of cultures with dex, stained with non-specific myeloma IgGs. The black in the upper left corner is a portion of a bone nodule. (B) The same cultures as in (A), but stained with ED2 mAb; a positively stained macrophage colony can be seen. (C) Control cells for the same experiment as above, but grown without dex, stained with non-specific myeloma IgGs. In addition to the stromal cells, many small unstained hemopoietic cells can be seen - these appear dark when stained with control antibodies (non-specific IgGs) and/or second antibody alone or without any antibodies. (D) The same cultures as in (C), but stained with ED2 mAb; no positive colonies are seen. All magnifications x120.
Figure 2.7 Flow cytometry: frequency histogram vs fluorescent intensity. (A) and (E) Controls stained with non-specific myeloma IgGs, for cells grown without dex and with dex, respectively, in the same experiment. (B) and (F) Staining with RBM 211.13 for AP in cells grown without dex and with dex, respectively. (C) and (G) Staining with ED2 (macrophages) in cells grown without dex and with dex, respectively. (D) and (H) Staining with MRC OX-1 (leukocytes) in cells grown without dex and with dex, respectively. All cells were collected for analysis at d. 21 of culture.
2.4 Discussion

In this paper, we provide quantitative evidence that RBM stromal populations grown under conditions promoting osteogenesis and bone formation comprise a heterogeneous mixture of cell types. We confirmed that the addition of dex, similar to its effects in other osteogenic models (Canalis, 1983; Bellows et al., 1986) stimulates osteogenesis and the formation of bone-like tissue or bone nodules in vitro from adult RBM stromal cells (Maniatopoulos et al., 1988; Kasugai et al., 1991; Leboy et al., 1991). The presence of other morphologically distinguishable cell types in stromal cultures making bone has been noted on occasion but not carefully investigated. For example, previously we reported that the predominant cells under similar culture conditions with dex were of spindle-shaped morphology (fibroblastic) or were pleomorphic and the majority did not stain for AP activity (McCulloch et al., 1991). More recently, in a molecular and immunohistochemical study of osteoprogenitor differentiation, we confirmed that only a proportion of the cells in RBM stromal cultures are osteogenic (Malaval et al., 1994). In this paper, we characterized and quantified the presence of cells of the monocyte-macrophage lineage and adipocytes, both lineages known to be present in stromal cultures of mouse and human (Dexter, 1982; Wang and Wolf, 1990), but not previously identified in adult RBM stromal cultures undergoing osteogenesis.

Dex significantly increased the numbers of AP positive colonies and cells in RBM stromal cultures, a finding consistent with reports that dex increased AP enzyme levels in the mouse BM stromal cell line, MBA-15 (Benayahu et al., 1989), and in other marrow stromal-derived cell lines (Benayahu et al., 1991). Interestingly, we found that both the percentage area of the culture dish covered by AP positive colonies and bone nodule production plateaued in the RBM stromal cultures at similar times, but only a small proportion (about 1/4) of AP positive colonies were coincident with bone nodules. Likewise in chick BM stromal cultures, only a proportion, albeit higher than in rat (55% versus 25%) of the AP positive
cell population contributes to osteogenesis (Kamalia et al., 1992). This low proportion of bone AP to total AP colonies could be due to AP positive osteoblastic cells being present, but either lacking the ability to proliferate sufficiently to form a bone nodule and/or being at stages of differentiation which they cannot form bone in vitro (see also discussion in Malaval et al. (Malaval et al., 1994)). Alternatively, while AP is a well-known marker of bone cells, it is not exclusive to them, and the presence of other, non-bone, AP positive lineages is possible. For example, several preadipose cell lines are AP positive (Udagawa et al., 1989; Benayahu et al., 1991; Dorheim et al., 1993).

Only low numbers of adipocyte colonies were seen under our culture conditions. This is in contrast to other systems where production of adipocytes is stimulated by the addition of glucocorticoids (Dorheim et al., 1993; Bellows et al., 1994). Bennett et al. (Bennett et al., 1991) have suggested that there is a bipotential progenitor in rabbit BM stroma which can differentiate in an adipocytic direction or along the osteogenic pathway. When Beresford et al. (Beresford et al., 1992) grew adult rat marrow stromal cells in the presence of FCS and dex, both adipocytic and osteogenic cells differentiated, but an inverse relationship between adipocytic and osteogenic cells was seen. The latter authors showed that with continuous exposure to dex in both the primary and secondary cultures, the differentiation of osteogenic cells predominated, whereas the differentiation of adipocytes predominated when dex was present in secondary culture only. Although dex was present only in the secondary cultures in our experiments, few adipocyte colonies were seen, suggesting that other factors in our culture conditions favor differentiation of osteogenic cells over adipocytes, whether there is a bipotential adipo-osteogenic cell present or not (for further discussion, see Bellows et al. (Bellows et al., 1994). For example, Beresford et al. (Beresford et al., 1992) used female rats (we used male), an initial period in the primary where the medium was not changed for 5 - 6 d. (we changed medium 3 times/week), and a shorter secondary culturing time of 8 - 10 d. (versus our time of 21 d.), all conditions that could markedly affect the subpopulation
make-up of the cultures and influence the ability of multiple cell populations to interact and/or differentiate.

As previously mentioned, Simmons et al. (Simmons et al., 1991) reported that primary RBM stromal cultures contained 10% "residual marrow components" of macrophagic/granulocytic leukocytes. Maniatopoulos et al. (Maniatopoulos et al., 1988) also noted the presence of small, mononuclear round cells in their primary cultures, and attributed them to the hemopoietic component of BM, but did not comment on them in the secondary cultures, in which they analyzed osteogenesis. However, the repeated medium changes in the 7 d. primary culture period adapted from Maniatopoulos et al. (Maniatopoulos et al., 1988) and used here is thought to reduce hemopoietic contamination while expanding the numbers of CFU-F and decreasing cellular debris. Clearly, the hemopoietic population recognized by MRC OX-1 (Cedarlane), which detects leukocyte common antigen, is present in the stromal layer of cells in cultures grown both in the absence and presence of dex. However, dex reduced the fraction of hemopoietic cells that express this antigen in these cultures. Of interest, the majority of the hemopoietic cells present in dex-containing cultures are macrophages, while a much smaller proportion are macrophages in the absence of dex. These other MRC OX-1 positive cells appear not to label with MRC OX-22, suggesting that they are not lymphocytes, and by histochemical stains appear not to be neutrophils. They may be monocytes or more primitive hemopoietic cells, however, the paucity of specific mAbs to rat hemopoietic cells, especially when compared with those available for mouse and human, currently hinders their identification.

The finding that macrophage production is stimulated under conditions also stimulating bone formation is interesting. While macrophages have been reported in murine BM secondary cultures (Wang and Wolf, 1990), we are unaware of any definitive identification of hemopoietic cells in secondary cultures of RBM stroma grown under conditions favoring
bone formation. Notably, clearly concomitant with stimulating the differentiation of osteoprogenitors, dex stimulated the maturation of the monocytic lineage into macrophages in our cultures, possibly while inhibiting proliferation of more primitive precursors or altering the proportion of other lineages in the hemopoietic fraction. An alternate explanation could be that dex stimulates osteogenic cells or other stromal cells to produce factors which indirectly stimulate macrophage production. Murine osteoblastic cells are known to produce M-CSF and GM-CSF (Felix et al., 1988; Horowitz and Jilka, 1992), and recent reports describe the detection of mRNA transcripts for IL-1β, IL-6, IL-8, and TGF-β1,2,3 in normal human bone (Birch et al., 1993) and for IL-5, IL-6, IL-7, M-CSF, GM-CSF, and TGF-β1 in the MC3T3-E1 cell line and rat primary osteoblastic cultures (Greenfield et al., 1993). As well, mouse BM stromal cells produce stimulators such as M-CSF and GM-CSF (Benayahu et al., 1992), and constitutively produce mRNAs for CSF-1, GM-CSF, kit ligand, and IL-6 (Kittler et al., 1992). Thus it appears that osteoblasts or other stromal cells secrete many factors that could affect the hemopoietic lineages, including macrophage production. Of course, the inverse is also possible: with dex altering hemopoietic lineages, stimulating production of mature macrophages and altering cytokine levels in such a way that stimulation of bone nodule production results. As well, the differentiation of monocytes into macrophages could result in the loss of production of inhibitory factors. For example, IL-1β, which has biphasic effects on bone formation but is inhibitory when present continuously during the differentiation sequence (Ellies and Aubin, 1990), is secreted by monocytes, but its secretion decreases with differentiation into macrophages (Kreutz et al., 1993). In any case, it appears crucial to identify the cell populations present in this system, as the effects of any factor on bone formation in this system may be direct or indirect and mediated through the other cell lineages present.

In RBM stromal populations, the culture conditions used to promote growth and differentiation of AP positive colonies, a subpopulation of which includes osteoprogenitor
cells that differentiate to form bone nodules (colonies of bone), concomitantly reduce the proportion of hemopoietic cells expressing leukocyte common antigen, while promoting the growth and differentiation of macrophages. These data show conclusively for the first time the presence of macrophages in RBM stromal cultures grown under conditions favouring bone formation, extend the information on the subpopulation make-up and heterogeneity under these culture conditions, and contribute to an understanding of the multitarget phenomenon observed in osteogenic differentiation in RBM stroma.
CHAPTER 3

Cell Sorting Enriches Osteogenic Populations in Rat Bone Marrow Stromal Cell Cultures

This chapter has been accepted for publication in Bone (December, 1997) as an original paper with the above title (Alexandra Herbertson and Jane E. Aubin)
3.1 Introduction

While a number of clonal, established and immortalized bone-derived cell lines have been isolated, many of these appear to show anomalies in aspects of their phenotype and regulation by hormones and growth factors (for review, see (Aubin et al., 1993)). It is therefore of considerable importance to identify the normal osteoprogenitors in populations freshly isolated from tissue. Cultures of whole marrow stromal cells were first shown to contain cells with the capacity to form bone when they were transplanted in vivo in diffusion chambers by Friedenstein and colleagues (Friedenstein et al., 1968). This has been confirmed repeatedly in vivo with BM cells in diffusion chambers (for example, see (Ashton et al., 1984; Owen, 1988)) and, more recently, conditions have been established in which bone formation is observed in vitro in cultures of BM stromal cells from diverse species including adult rabbit (Tibone and Bernard, 1982), adult male (Maniatiopoulos et al., 1988) and female (Leboy et al., 1991) rat, chick embryo (Kamalia et al., 1992), neonatal pig (Thomson et al., 1993), adult mouse (Van Vlasselaer et al., 1993), and adult human (Vilamitjana-Amedee et al., 1993). In stroma (McCulloch et al., 1991), as in calvarial populations (Bellows and Aubin, 1989; Bellows et al., 1990), the bone nodule assay can be used as an indirect functional assay of the osteoprogenitor, the end product being a nodule of osteoblasts/osteocytes embedded in a mineralized matrix characterized as bone on the basis of light and phase contrast microscopy, histochemistry including von Kossa staining, scanning electron microscopy, and transmission electron microscopy (Tibone and Bernard, 1982; Maniatiopoulos et al., 1988). The RBM stromal cell system has been well characterized in terms of morphological characteristics of differentiating osteoblasts and their matrix (Maniatiopoulos et al., 1988; Satomura and Nagayama, 1991), in terms of molecular and immunohistochemical details of the expression of markers associated with bone formation (Kasugai et al., 1991; Malaval et al., 1994), and with respect to the effect of exogenous agents on bone formation (Ogiso et al., 1991; Zhang et al., 1991; Notoya et al., 1994; Tenenbaum et al., 1995). However, in RBM stromal cell cultures, only a small
proportion of the cells are osteogenic, and the presence of other morphologically distinguishable cell types has been reported (Malaval et al., 1994; Herbertson and Aubin, 1995). For example, in these culture conditions, while some osteoprogenitors can be assayed in the absence of dex, the assayable number increases markedly in dex, concomitantly with the growth and differentiation of macrophage colonies (Herbertson and Aubin, 1995). The presence of multiple cell types in RBM stromal populations and their varying proportions from one isolation to another despite rigorous attention to isolation protocol may account for the considerable variation in the absolute number of bone nodules formed in different experiments, and may complicate interpretation of cytokine and hormone effects as direct or indirect on the osteoprogenitors present. The presence of these multiple subpopulations suggests a need for a method which can purify the osteogenic component, including osteoprogenitors, from other lineages in the RBM stroma. To remove the majority of well-differentiated hematopoietic cells when culturing, centrifugation or adherence techniques are commonly used. Maniatopoulos et al. (1988) noted in their primary cultures the presence of small hematopoietic cells and the repeated medium changes in the 7 d. primary culture period were used to reduce their presence (Maniatopoulos et al., 1988; Kamalia et al., 1992) and cellular debris while expanding the CFU- F (Maniatopoulos et al., 1988), of which osteogenic colonies are a subgroup. However, it is also known that hematopoietic stem cells are present at low frequency in the adherent marrow cell fraction (Kiefer et al., 1991) and their subsequent development is guided by the existing culture conditions. Agents for hemolysis and density gradients are other commonly used strategies in other systems for decreasing hematopoietic contamination (Falla et al., 1993). Nijweide and colleagues reported selective cyanide killing of monocyte-macrophage lineage cells in mouse marrow stromal cell cultures (Modderman et al., 1994); in our hands, no specific killing effects were achievable with this method in RBM stromal cultures (unpublished observations). Macrophage and endothelial cell components can also be separated from the fibroblast-like population by their phagocytic properties (Stein and Stein, 1980; Pitas et al.,
1981; Yanai et al., 1991) and magnetic bead separation techniques based on phagocytosis (Zhang et al., 1995) have been reported. Flow cytometric analysis based on phagocytosis of fluorescently labeled acetylated low density lipoprotein was found to be less effective in identifying macrophages compared to mAB labelling (unpublished observations).

In addition to the strategies used to decrease the hematopoietic population, there has also been investigation into methods to enrich selectively for osteoblast populations from stromal populations. Selective adherence has been one approach, as summarized in Chapter 1, Section 1.5. Increasingly, the availability of mAbs recognizing subpopulations of osteoblasts (Aubin and Turksen, 1995) is providing means for antibody-based methods for purification. AP expression has been reported previously to be useful in fractionation of osteoblastic populations into an AP-positive population and an AP-negative population in the rat calvaria system; interestingly amongst the former were dex-independent progenitors and in the latter were progenitors which required dex to differentiate (Turksen and Aubin, 1991). AP expression has also been used for flow cytometric sorting in female RBM stromal cell cultures, but production of a mineralized matrix after sorting was only described in cells which were AP-negative at the time of sorting (Rickard et al., 1994). In this paper we describe flow cytometric sorting of RBM stroma based on AP expression, with resultant enrichment for osteoprogenitors capable of dividing and differentiating to form osteoblasts which synthesize bone.
3.2 Materials and Methods

Cell culture and plating

BM stromal cells from the femora of young 40 - 43-day-old, 110 - 120 gram, male Wistar rats were cultured essentially as described previously (Maniatopoulos et al., 1988), see Chapter 2. After 7 d., adherent cells were recovered in a single cell suspension, then either counted electronically on a Coulter Counter (Coulter Electronics, Hialeah, FL) and plated at densities between 2 x 10^4 and 4 x 10^4 cells per 35 mm dish (Falcon) (unsorted controls), or labelled and processed through the sorter (see below) prior to plating. After plating, cells were cultured for an average of 21 d., then stained (AP/von Kossa/toluidine blue, αNBE, and Sudan IV) as previously described in Chapter 2.

Flow cytometry

Cells were recovered from flasks or dishes were passed through a syringe with a 22 gauge needle, and centrifuged. The cells were resuspended in fresh growth medium and incubated at 37°C for 30 - 60 minutes to allow the cells to recover from the trauma of the procedure. Cells were centrifuged then resuspended in sterile primary mouse anti-rat antibodies diluted in 1.5% BSA in PBS: mouse myeloma Ig's (Organon Teknika Co., West Chester, USA) for controls, RBM 211.13 for AP (Turksen et al., 1992), MRC OX-1 (Cedarlane Laboratories Ltd., Hornby, Canada) for leukocyte common antigen (Sunderland et al., 1979), ED2 (Serotec Canada, Toronto, Canada) for macrophages (Dijkstra et al., 1985), or RECA (Medac GmgH, Hamburg, Germany) for rat endothelial cells (Duijvestijn, 1992), and incubated at 37°C for 30 minutes. After incubation, cells were washed twice then incubated with FITC-conjugated sheep anti-mouse antibodies (Serotec) diluted in 1.5% BSA in PBS for 30 minutes at 37°C and then washed with PBS twice. Cell suspensions were then placed on ice and flow cytometric sorting was begun immediately; the sorting process took on average 2 - 4 hr. Immediately before analysis, cells were passed through 22 gauge needles
to remove any cell clumps. For flow cytometric counting, propidium iodide was added and populations were double-gated for low propidium iodide staining and size. For cell sorting, samples were also gated for size but propidium iodide was added only to a small control sample to determine cell vitality and plating numbers were varied accordingly (one sorting trial with small amounts of propidium iodide - 5 μl/ml cell suspension - left in demonstrated its toxicity in these populations). Samples were run on a EPICS Cell Sorter (Coulter) equipped with an Argon laser operated at 488 nm for fluorescence excitation. Fluorescence was detected with a 525 nm band pass filter (± 10 nm). Sorting regions were set a minimum of 3% away from the border of the 1% negative control region to exclude overlapping populations. Percentage of positive cells (FITC versus Count) was monitored throughout the sort as a check for antigen capping, although this was not observed to be a problem during the sorting periods. Sorted cells were collected in tubes with α-MEM containing 30% fetal calf serum and antibiotics as above; they were then diluted in medium as above and plated accordingly. For sort controls, cells were stained as above, one gate expanded to include 100% of the population, and cells were passed through the machine prior to collection.

Quantitation

Quantitation of colony types involved identification of AP-positive colonies by staining (red) as above, identification of bone nodules by black von Kossa stained mineral deposits surrounded by cuboidal AP-positive stained cells, monocytes/macrophages by αNBE staining (dark brown), and adipocytes by (red) Sudan IV staining and morphology. For quantitation, only foci containing >10 positively stained cells (approximately three population doublings) were classified as colonies; both colony counts and grid point counts for proportion of the culture dish covered were done. For statistical analysis, the Student t test (unpaired, double-sided) was used to determine levels of significance, unless the standard deviations differed significantly, in which case a Welch t test was used.
3.3 Results

When RBM stromal cells were analyzed by flow cytometry after either 7 d. primary culture or after 7 d. primary and 18 d. secondary culture, they exhibited a broad distribution of cell sizes consistent with the multiple hematopoietic and mesenchymal subpopulations present (Herbertson and Aubin, 1995), necessitating the use of both log forward scatter (related to cell size) and log side scatter (related to cell granularity) in analysis and sorting parameters. In both counting and sorting experiments, the populations were first gated for size: using log forward scatter, the gated area was set to remove small fragments and debris or discard any obvious cell clumps, with a lower limit consistent with platelet size (approximately 2 μm) and an upper limit consistent with large cultured cells (approximately 30 μm). On the basis of fluorescence, control cells incubated with mouse myeloma Ig's as the primary antibody were gated to 1.0% (see Figure 3.2A) to eliminate 99% of the background autofluorescence (a normal characteristic of all viable mammalian cells (Aubin, 1979) and marked in some BM stromal cells (Keller et al., 1993)) and any non-specific staining (see Figures 3.1A and 3.1B, and control in Figure 3.2). With these conditions, RBM populations labelled with the anti-rat AP antibody (RBM 211.13) displayed a broad range of labelling intensity (see Figure 3.1C).

In RBM stromal cells grown for 7 d. primary and then 18 d. secondary culture, multiple cell lineages are represented, with subpopulations varying according to the presence or absence of dex, as we previously reported (Herbertson and Aubin, 1995). Typically, more cells labeled with RBM 211.13 (for AP) when RBM stromal cells were grown in the presence (74.7%) than in the absence of dex (13.9%) (see Figure 3.2A and 3.2B). In contrast, cells staining for MRC OX 1 (for leukocyte common antigen) were present at lower frequency in cultures with dex (2.8%) than without dex (21.2%). A small percentage of cells stained positively with ED2 (anti-macrophages) only in the cultures with dex (2.0%). RECA, an
antibody specific for rat endothelial cells (Duijvestijn et al., 1992), stained few cells in either cultures with (1.1%) or without dex (0.9%), representing < 1% of cells over background. In multiple experiments, by either flow cytometry or immunohistochemical staining (data not shown), negligible numbers of cells stained with RECA, suggesting that endothelial cells are present in low numbers in RBM stromal cell cultures under these growth conditions.

For cell fractionation after 7 d. primary culture, sorting gates were set to capture cells either APhigh (cells intensely labeled with RBM 211.13) or APlow (clearly negative for RBM 211.13 labeling) as indicated in Figure 3.1; in this experiment 31.2% of cells were in the former category and 32.7% in the latter (see Figures 3.1B and 3.1D). APhigh, APlow, or control cells were plated to determine osteoprogenitor distribution and frequency. Similar to results we reported previously with flow cytometric sorting of fetal rat calvarial populations (Turksen and Aubin, 1991), and consistent with reports that murine osteoblastic cells (Van Vlasselaer et al., 1994) and stroma initiating cells are depleted by passage through a flow cytometer (Deryugina and Muller-Sieburg, 1993; Deryugina et al., 1995), approximately 50% (on a per plated cell basis) of nodule forming cells are lost during processing/sorting when compared to unsorted controls. In some experiments, we therefore added an additional control, measuring cell viability with propidium iodide at the end of labelling manipulations and then adjusting plating densities to compensate for reduced cell viability. The number of AP-positive colonies was enriched in the APhigh fraction of RBM cells compared to either the unfractionated cells or the APlow fraction, whether or not the sorted cells were cultured in the presence or absence of dex (see Table 3.1). However, since numbers of bone nodules and macrophage colonies are low in cultures without dex (Herbertson and Aubin, 1995) and even in the APhigh fraction bone nodule numbers were significantly higher (p ≤ .05) when cultured with dex versus without dex, and since trends in bone nodule or macrophage colony numbers were not significant in minus-dex cultures, subsequent results are reported only for dex-containing cultures. Table 3.2 summarizes 5
separate experiments. It is clear that within the AP\textsuperscript{high} fraction the number of AP-positive colonies and osteoprogenitors (giving rise to bone nodules) were significantly enriched compared to control, but as we reported earlier, bone colonies comprise only a small proportion of the total AP-positive colonies (Herbertson and Aubin, 1995). The increase in osteoprogenitor frequency ranged from approximately 2 to 100 fold (see Table 3.3). The fold increase in osteoprogenitor frequency did not appear to correlate with either the initial percentage of cells positive for AP in the culture at the time of sorting, nor did it appear to correlate with any fold change in macrophage colonies (as determined by αNBE staining) (see Table 3.3). In addition, the fold increase in osteoprogenitor frequency did not appear to correlate with the post-sorting purity check; both experiments B and C in Table 3.3 had fold increases of 1.7x but different post-sorting purities (B post-sorting purity was 95.8%, C post-sorting purity was 96.5%), while in experiment D in Table 3.3 the post-sorting purity was lower, 93.5%, but the fold increase was higher at 3.5x.

Concomitantly with the significant enrichment of osteoprogenitors, the AP\textsuperscript{high} fraction of cells was usually significantly depleted for macrophages (in 4/5 experiments listed in Tables 2 and 3). In the anomalous experiment, experiment C in Table 3.3, macrophage numbers were very high in the unfractionated control; while the total number of macrophage colonies increased in the AP\textsuperscript{high} fraction, in this experiment the number of large (>100 cells) macrophage colonies in the AP\textsuperscript{high} fraction (3.3 ±1.5 colonies) significantly decreased (p ≤ .01) compared to the unfractionated control (13.0 ± 2.0 colonies). The apparent increase in macrophage colonies is likely due to the decrease in number of large macrophage colonies, which tend to overgrow and obscure smaller colonies that may also be present. Thus, although total colony number suggests otherwise, in experiment C the actual number of individual macrophages probably decreased. In contrast, macrophages appear to be consistently enriched in the AP\textsuperscript{low} fraction of cells, a fraction with no consistent changes from control in either AP-positive colonies or osteoprogenitors (see Table 3.2).
Very low numbers of adipocyte colonies form in RBM stromal cell cultures under these growth conditions (Herbertson and Aubin, 1995). Of the 3 experiments in which adipocyte colonies were quantitated, 2 had fat colonies present in large enough numbers (> 4 colonies per dish in the unfractionated cells) to be analyzed statistically. Within the AP\textsuperscript{high} fraction of cells, adipocyte colonies were significantly depleted, while they were significantly enriched in the AP\textsuperscript{low} fraction (see Table 3.2 and Figure 3.3).

While enrichment of bone nodules and AP-positive colonies is seen within the AP\textsuperscript{high} fraction, it is also apparent that this fraction is still a mixed population. For example, culture dishes are not completely covered with AP-positive colonies (see Table 3.1), reflecting the presence of AP-negative colonies, and while the macrophage colony number is generally decreased, some are still present (see Table 3.3). As mentioned earlier, post-sort purity checks (re-analyzing the sorted population on the flow cytometer) of the AP\textsuperscript{high} cells showed that this fraction was minimally 93 - 97% pure; thus some of the non-AP-positive colonies may reflect the 3 - 7% "contaminating" cells. Post-sort purity checks of the AP\textsuperscript{low} cells showed that this population was minimally 99% pure, reflecting the relative ease in excluding a highly stained cell.

MRC OX-1 is a mAB reactive against leukocyte common antigen, which is known to be present on > 95% of thymocytes, BM cells, and thoracic duct lymphocytes (Sunderland et al., 1979). Two sorting trials with this antibody were performed to see if they yielded "cleaner" RBM stromal populations, i.e., populations better depleted of hematopoietic cells. While the removal of the majority of the hematopoietic cell fraction increased AP-positive colony and bone nodule numbers, macrophages were also present in numbers equal to or greater than those in unfractionated control populations (results not shown).
Previous work in our laboratory has shown that in RBM stromal populations osteoprogenitor expression, as determined by bone nodule formation, does not follow a linear relationship in limiting dilution analysis until very high cell densities are reached, suggesting cooperativity of cell types within the population and a multi-target phenomenon (Aubin et al., 1990) (Aubin, 1997, submitted). When we analyzed osteoprogenitor frequency/nodule number versus plating density in control versus APhigh and Aplow populations, osteoprogenitor expression still followed a non-linear relationship (Figure 3.4A). Similarly, but not to the same degree, the αNBE positive macrophage colonies appear to follow a non-linear relationship with decreasing plating densities (Figure 3.4B).
Table 3.1

Percentage of 35 mm culture dishes covered by AP-positive colonies (± one standard deviation) as determined by grid point counts.

The experiment designation (A or B) refers to the same experiments as seen in Table 3.3. Comparisons for significance were done between sorted samples and the corresponding control with or without dex.

T tests significance levels:  * p ≤ .05;  ** p ≤ .01;  *** p ≤ .001.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Control</th>
<th>High RBM 211.13 Expression</th>
<th>Low RBM 211.13 Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ or - Dex</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>A</td>
<td>89.9 ± 2.8</td>
<td>97.5 ± 0.6***</td>
<td>75.2 ± 3.7***</td>
</tr>
<tr>
<td>B</td>
<td>36.0 ± 5.8</td>
<td>51.1 ± 0.5**</td>
<td>35.3 ± 2.2</td>
</tr>
</tbody>
</table>
Table 3.2

Net changes (enrichment/depletion) in various colony types in AP high and AP low fractions, compared to unfractionated controls. All cultures contained dex after plating. Changes indicated by the arrows were significant at minimally $p \leq .05$ as determined by t tests, or the trend seen is indicated.

<table>
<thead>
<tr>
<th></th>
<th>AP High</th>
<th>AP Low</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline Phosphatase</td>
<td>↑ 5/5</td>
<td>↓ 3/5 (trend ↓ 1/5) ↑ 1/5</td>
</tr>
<tr>
<td>Bone Nodules</td>
<td>↑ 5/5</td>
<td>↓ 3/5 ↑ 2/5</td>
</tr>
<tr>
<td>αNBE + Colonies</td>
<td>↓ 3/5 (trend ↓ 1/5) ↑ 1/5</td>
<td>↑ 4/5 (trend ↑ 1/5)</td>
</tr>
<tr>
<td>Fat Colonies</td>
<td>↓ 2/2</td>
<td>↑ 2/2</td>
</tr>
</tbody>
</table>
Table 3.3

Five separate RBM stromal cell sorting experiments, detailing the changes (enrichment/depletion) when cells were sorted on the basis of high AP expression, then cultured in the presence of dex. The percentage of cells positive for RBM 211.13 expression at the time of sorting (initial %) is listed, along with the numbers (± one standard deviation) of bone nodules (BN) and macrophage (αNBE +) colonies. The fold changes between control cells and sorted cells for BN (BN Δ) and macrophages (αNBE + Δ) is also shown (↑ = increased relative to control; ↓ = decreased relative to control).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>initial %</th>
<th>BN (control)</th>
<th>BN (High)</th>
<th>BN Δ</th>
<th>αNBE + (control)</th>
<th>αNBE + (High)</th>
<th>αNBE + Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>20.2</td>
<td>4.5 (± 1.3)</td>
<td>8.3 (± 2.2)</td>
<td>↑ 1.8</td>
<td>5.3 (± 0.9)</td>
<td>2.7 (± 2.1)</td>
<td>↓ 2.0</td>
</tr>
<tr>
<td>B</td>
<td>11.5</td>
<td>10.8 (± 0.8)</td>
<td>18.0 (± 4.2)</td>
<td>↑ 1.7</td>
<td>31.3 (± 1.5)</td>
<td>23.5 (± 2.1)</td>
<td>↓ 1.3</td>
</tr>
<tr>
<td>C</td>
<td>30.2</td>
<td>480.8 (± 46.7)</td>
<td>815.3 (± 27.5)</td>
<td>↑ 1.7</td>
<td>298.0 (± 15.1)</td>
<td>349.0 (± 14.4)</td>
<td>↑ 1.2</td>
</tr>
<tr>
<td>D</td>
<td>38.8</td>
<td>30.4 (± 7.8)</td>
<td>105.2 (± 17.3)</td>
<td>↑ 3.5</td>
<td>148.0 (± 13.9)</td>
<td>37.2 (± 6.3)</td>
<td>↓ 4.0</td>
</tr>
<tr>
<td>E</td>
<td>16.9</td>
<td>1.0 (± 1.7)</td>
<td>83.7 (± 11.7)</td>
<td>↑ 84</td>
<td>39.0 (± 7.1)</td>
<td>23.0 (± 2.8)</td>
<td>↓ 1.7</td>
</tr>
</tbody>
</table>
Figure 3.1
Flow cytometric sorting. (A) and (C) are frequency (count) histograms versus cell fluorescence (FITC) intensity for control (incubated with mouse myeloma Ig's as the primary antibody) and RBM 211.13 (AP) stained RBM stromal populations respectively. In (A), the control cells have the lower cut-off of cursor 'a' set to 1.0%. In (C), the number of cells in the area 'a' represents those positively stained for RBM 211.13 (38.8%). (B) and (D) are forward scatter log (FS log; representing size) versus FITC intensity contour maps for control and RBM 211.13 stained populations respectively. The sorting gates were set to capture cells with either high (gate 'c') or low (gate 'b') expression of RBM 211.13. In (B) gate 'b' contains 66.4% and gate 'c' contains 1% of the total population, whereas in (D) gate 'b' contains 32.7% and gate 'c', which has been shifted right relative to the control, contains 31.2% of the total population.
Figure 3.2 Legend

Flow cytometric analysis. (A) and (B) are frequency (count) histograms versus cell fluorescence (FITC) intensity in RBM stromal cell cultures grown in secondary cultures for 18 d. without or with dex, respectively. From top to bottom: RBM stromal cells are stained with control mAb (incubated with mouse myeloma Ig's as the primary antibody) and RBM 211.13 (anti-AP), MRC OX-1 (anti-LCA), ED2 (anti-macrophage), and RECA (anti-rat endothelial cell antigen).
Figure 3.3

Adipocyte colony numbers for RBM stromal populations sorted according to RBM 211.13 (AP) expression. Data is from the experiment designated "D" in Table 3. T tests show that adipocyte colonies are significantly lower (p ≤ .01) in AP high (high RBM 211.13 binding), and are significantly higher (p ≤ .01) in AP low (low RBM 211.13 binding) populations.
Figure 3.4 Legend

Bone nodules (A) or αNBE colonies (B) versus plating density for RBM stromal populations sorted according to RBM 211.13 (AP) expression. Data are from the experiment designated "C" in Table 3.3. In (A), t tests show that osteoprogenitor (capable of giving rise to a bone nodule) numbers are significantly lower (p ≤ .01) in the AP low (low RBM 211.13 binding), and are significantly higher (p ≤ .01) in the AP high (high RBM 211.13 binding) at all densities from 2 x 10^4 to 2.5 x 10^3. In (B), t tests show that αNBE positive colony numbers are significantly lower (p ≤ .01) in AP low (low RBM 211.13 expression) populations only at the 2 x 10^4 density, and are significantly higher (p ≤ .05) in AP high (high RBM 211.13 expression) populations at densities from 2 x 10^4 to 2.5 x 10^3.
3.4 Discussion

Previous work from our lab has shown the presence of multiple cell types in RBM stromal populations, including cells of the monocyte-macrophage lineage, fibroblasts, and adipocytes (Herbertson and Aubin, 1995). Notably the addition of dex, concomitant with stimulating the differentiation of osteoprogenitors to bone nodule forming cells, stimulated the maturation of the monocytic lineage into macrophages in our cultures, possibly while inhibiting proliferation of more primitive precursors (Herbertson and Aubin, 1995). In cultures with dex, macrophages appear to make up the majority of the hematopoietic cells present (Herbertson and Aubin, 1995). Despite rigorous attention to cell isolation techniques, the proportions of hematopoietic versus mesenchymal subpopulations present in RBM stroma appear to vary from one isolation to another and may account for the considerable variation in the absolute number of bone nodules seen in different experiments. Consistent with the hypothesis that heterotypic cell-cell interactions influence osteogenesis in the RBM system (Aubin et al., 1990; Aubin, 1997, submitted), the presence of these multiple subpopulations also complicate the interpretation of direct versus indirect cytokine effects in BM systems (Thomson et al., 1993; Herbertson and Aubin, 1995). Notably, in our fractionated populations, more than one cell type remains limiting in the populations, suggesting that cell-cell interactions still appear to influence osteoprogenitor differentiation and nodule formation; in addition, it is possible that homotypic cell interactions, i.e. "community effects" may also play a role as hypothesized recently based on limiting dilution and other frequency analyses (Aubin, 1997, submitted).

Clearly, in the AP\textsuperscript{high} fraction of cells we enriched for osteoprogenitors which divide and differentiate to give rise to bone nodules, although the resultant cultures still comprised a mixture of lineages. This mixture of lineages may reflect the 3 - 7 % "contaminating" cells in the AP\textsuperscript{high} fraction, and the 1% "contaminating" cells in the AP\textsuperscript{low} fraction. Although
flow cytometric sorting can yield populations of high purity. 100% purity is only ever approximated unless single cell cloning methods are added to the isolation step. Given the large range of cell sizes seen in the RBM stromal populations, our sort gates were accordingly set wider, which would also tend to reduce the stringency of the sort. Nevertheless, the 93 - 97% purity obtained in our sorting experiments are similar to those achieved by an improved immunopanning technique applied to isolate CD 34+ cells from human BM (93.5% ± 3.4 % purity), and better than the 57 - 80% purities obtained in some other immunopanning procedures (Cardoso et al., 1995). Multiple antibody sorting steps and an imposition of single cell collection methods may help to reduce the presence of the contaminating cells further.

The mixed nature of the AP<sub>high</sub> population could result not only from limitations in the efficacy of sorting, but also from cells that have reverted from AP-positive osteoblastic cells to a more immature, AP-negative phenotype, or the presence of other, non-bone, AP-positive lineages. The presence of AP-positive colonies and osteoprogenitors (giving rise to bone nodules) in the AP<sub>low</sub> fraction is likely representative of the presence of immature progenitors which, at the time of sorting, are not yet expressing AP (see also (Turksen and Aubin, 1991)). While AP is a well known marker of the mature osteoblast lineage, AP is also a cytochemical marker of 'reticular' cells in marrow which make up a large proportion of the marrow stroma (Westen and Bainton, 1979; Cattoretti et al., 1991): these cells have often been postulated to be preadipocytes (Bianco et al., 1988; Ross et al., 1991) which are known to be AP-positive (Udagawa et al., 1989; Benayahu et al., 1991; Dorheim et al., 1993). However, in our stromal cell cultures with the highest numbers of fat colonies present in the controls, fat colony number was decreased in the AP<sub>high</sub> and increased in the AP<sub>low</sub> fraction of cells. Although isolated brain capillary endothelial cells express high AP activity which decreases with cell culturing (Meyer et al., 1990), bone endothelial cells have been reported to express only low levels of AP (Rickard et al., 1995). We would thus expect
these not to be found in the AP<sup>high</sup> fraction of cells, especially since direct quantitation of endothelial cells by endothelial specific antibodies suggested that they do not form a significant proportion of the RBM stromal cell cultures under our culture conditions.

While the finding that cells at different stages of the osteoblast lineage express antigenic determinants unique or distinctive to these stages is beginning to be useful for identification and isolation of osteoblastic cells at various stages of differentiation (Aubin and Turksen, 1995), we are still limited by a lack of specific antibodies definitely recognizing cells earlier than the preosteoblast. Haynesworth et al. (1992) reported isolation of antibodies that react on a subset of human marrow stromal cells and a variety of tissues in vivo, but do not label osteoblasts or osteocytes (Haynesworth et al., 1992). The authors concluded that the antibodies may recognize molecules present on early progenitors or stem cells and that the epitope(s) is lost during osteogenic differentiation (Haynesworth et al., 1992). STRO-1 recognizes human marrow stromal mesenchymal progenitors with the ability to form cells with the phenotype of fibroblasts, adipocytes, smooth muscle cells, and osteoblasts (Simmons and Torok-Storb, 1991; Gronthos et al., 1994). Very recently, HOP-26, a mAB against human marrow stromal osteoprogenitors, has been described (Joyner et al., 1997). HOP-26 labels an epitope present on osteoprogenitors before the induction of AP, but does not bind to osteoblasts, and its presence on the cell surface allowed enrichment of human marrow CFU-F including osteoprogenitors (Joyner et al., 1997). As yet, similar antibodies to early progenitors do not exist for the rat nor have the macromolecules recognized by these antibodies been identified to allow preparation of rat-specific reagents. Enrichment of relatively mature osteoblastic cells has also been reported. Immunopanning of human BM cells with osteocalcin (normally considered a marker of mature osteoblasts) and osteonectin has yielded some enrichment for cells with osteoprogenitor properties (Long et al., 1995). Van Vlasselaer and colleagues used immunopanning with mAbs to first deplete populations of hematopoietic cells, then used flow sorting with Sca-1 and wheat germ agglutinin binding
to enrich for osteoblastic cells from the 5-fluorouracil treated femoral BM of young adult mouse (Van Vlasselaer et al., 1994). Two mAbs (OB 7.3 and SB-5) that label the surface of chick osteocytes specifically have been used (with magnetic beads) to isolate relatively pure osteocytes from chick mixed bone cell populations (Van der Plas and Nijweide, 1992).

MAbs against AP have been raised by several labs (in our lab, RBM 211.13 was raised in an injection of RBM stroma (Turksen et al., 1992)); these label preosteoblastic cells as well as osteoblasts. In the rat calvarial system, AP expression has been used successfully to fractionate osteoblastic populations into AP-positive (highest 10% of cells) and AP-negative populations (Turksen and Aubin, 1991) and this report extends that work into the RBM stromal system. In the rat calvarial system, the fractionation resulted in osteoprogenitors being separated into two “classes”: one capable of expressing bone formation without exogenous dex stimulation (high AP-positive), and one expressing bone formation only in its presence (AP-negative) (Turksen and Aubin, 1991). These data are consistent with the hypothesis that these two populations of progenitor cells represent different stages of differentiation, with the latter representing a less mature cell than the former and requiring different regulatory signals for its expression than the latter (Turksen and Aubin, 1991). Given that few bone nodules form in RBM stromal cell cultures in the absence of dex, and the marked stimulation of bone nodule formation noted with dex, it is tempting to speculate that the majority of osteoprogenitors in stromal cultures, especially as assayed in the secondary cultures, may be of the relatively immature phenotype. In our experiments, we found few assayable osteoprogenitors (little bone nodule production) in the AP<sup>high</sup> fraction in the absence of dex, suggesting that RBM stroma contains largely dex-dependent osteoprogenitor populations and the possibility that dex is required later than when AP is first expressed.
Flow cytometric sorting of RBM stromal populations according to high or low AP expression is an effective technique for enrichment of AP colonies and osteoprogenitors which can divide and differentiate to give rise to bone nodules, and should prove useful for further studies of the nature of the osteoprogenitors and their regulation in this tissue.
CHAPTER 4

PDGF has Biphasic Effects on the Differentiation of Osteoprogenitors in Rat Bone Marrow Stromal Cell Populations

This chapter has been submitted for publication with the above title (Alexandra Herbertson and Jane E. Aubin)
4.1 Introduction

PDGF, 30 kDa disulphide bonded homo- or hetero-dimers of A and B chains which interact with the two PDGF receptors, α and β, appears to have broad roles in tissue development and repair (Bowen-Pope et al., 1991; Schatteman et al., 1992; Yan et al., 1993; Ponten et al., 1994; Yan et al., 1994). With respect to bone, normal human osteoblastic cells produce PDGF-A (Graves et al., 1989; Zhang et al., 1991) and PDGF α- and β-receptors (Zhang et al., 1991). PDGF B is reportedly not made by human osteoblastic cells (Graves et al., 1989; Zhang et al., 1991; Bilbe et al., 1996), although there is a recent report of its production by rat osteoblasts (Canalis and Rydziel, 1996) and mRNA for PDGF B has been detected in several human osteoblastic cell lines (MG-63 cell line, SaOs-2, TE-85) (Bilbe et al., 1996).

PDGF has been reported to affect both proliferation and aspects of the differentiated phenotype of osteoblastic populations in vitro. It increases proliferation in many osteoblastic model systems (fetal rat calvaria organ cultures (Canalis and Lian, 1988; Pfeilschifter et al., 1990), fetal rat calvaria cell cultures (Centrella et al., 1989; Centrella et al., 1991; Pfeilschifter et al., 1992), human osteoblastic cells (Graves et al., 1989; Gilardetti et al., 1991; Zhang et al., 1991; Abdennagy et al., 1992), mouse trabecular explant cells (Abdennagy et al., 1992) and the murine osteoblastic cell line MC3T3-E1 (Davidai et al., 1992)), consistent with its well known activity as a mitogen for other mesenchymal cells including fibroblasts, glial cells, and smooth muscle cells. However, whether it is osteoblastic cells alone responding in most of these models is not clear, as most experiments with primary cell cultures involve mixed cell populations, with fibroblasts and other stromal cells present which are also known to respond mitogenically to PDGF (Hirata et al., 1985). In this regard, PDGF was found to stimulate selectively fibroblast replication and function in fetal rat calvarial organ culture (Hock and Canalis, 1994).
The reported effects of PDGF on parameters of osteoblast differentiation have been diverse (no effect - (Canalis and Lian, 1988; Cassiede et al., 1996); positive effect - (Centrella et al., 1989; Pfeilschifter et al., 1990); negative effect - (Centrella et al., 1991; Pfeilschifter et al., 1992; Hock and Canalis, 1994)). These conflicting results may arise from several different sources, including the PDGF isoform used (there are isoform differences in ligand binding (Hart, 1990; Grotendorst et al., 1991)), and differing proportions of cell subpopulations with different PDGF receptor types (Hart, 1990), which mediate different responses (Westermark and Heldin, 1991; Allam et al., 1992; Coats et al., 1992; Pfeilschifter et al., 1992). The fact that different culture systems (e.g., fetal versus adult, organ/tissue versus cell, clonal versus mixed populations) and different culture conditions (e.g., serum supplementation or not) are being used to monitor osteoblast responsiveness to PDGF exacerbates the difficulty. The maturational status or differentiation of progenitor populations can also lead to changes in receptor levels: although PDGF α-receptor appears to be present throughout osteoblast development, the levels appear to increase with osteoblast differentiation (Liu and Aubin, 1996). The duration of exposure to PDGF may also be important; most investigations have used short exposure times (24 hr. (Canalis and Lian, 1988; Centrella et al., 1989; Zhang et al., 1991), 48 hr. (Pfeilschifter et al., 1990) or 72 hr. (Hock and Canalis, 1994)). Given earlier data with other factors such as EGF (Antosz et al., 1987; Aubin et al., 1992), which elicit biphasic effects depending upon the time and duration of exposure, and when during the proliferation/maturation sequence they were given, the effects of PDGF might be expected to vary depending on the treatment protocol. This was found to be the case, for example, when the effects of PDGF on proliferation and AP activity of marrow stromal cells were compared immediately after exposure to PDGF versus later (Cassiede et al., 1996). Finally, different parameters to measure osteoblast development have been used in different studies. For example, total protein synthesis, collagen synthesis, AP and/or osteocalcin production have variously been used as differentiation markers. While PDGF has been shown to increase collagen synthesis (Centrella et al., 1989; Pfeilschifter et al., 1990), the
collagen may be incorporated into a fibrous tissue matrix rather than a bone matrix (Pfeilschifter et al., 1990). Consistent with this, experiments which use more specific bone markers, such as AP (although not unique to the osteoblast lineage) and osteocalcin, suggest that PDGF inhibits bone formation in vitro (Centrella et al., 1992; Pfeilschifter et al., 1992; Hock and Canalis, 1994).

Osteoprogenitor cells present in the stromal population of young-adult RBM, cultured in medium containing ascorbic acid, β-glycerophosphate and dex, divide and differentiate to give rise to osteoblasts which synthesize bone-like tissue (Maniatopoulos et al., 1988); the number of bone nodules is quantifiable and provides a very specific, terminal differentiation product by which to measure the effects of PDGF on osteoblast differentiation. Given the established proliferation-differentiation sequence characteristic of the RBM model (see, e.g., Malaval et al., 1994), this system provides an opportunity to target specific events by varying the exposure times to PDGF. Further, BM stromal cells may mimic the in vivo environment, with its multiple subpopulations and different lineages present. For example, fibroblasts and macrophages, which we have previously reported to be present in RBM stromal cells grown under conditions which promote bone formation (Herbertson and Aubin, 1995), are also sources of and responders to PDGF (Bowen-Pope et al., 1991). Thus, while BM stromal cell cultures may recapitulate the complex cellular interactions that occur in vivo, establishing underlying mechanisms as direct or indirect on particular subpopulations can be difficult. Flow cytometric sorting of RBM stromal cells on the basis of AP expression, however, allows separation of the stroma into AP\textsuperscript{high} and AP\textsuperscript{low} populations. Within the AP\textsuperscript{high} fraction, the number of AP-positive colonies and osteoprogenitors (giving rise to bone nodules) are significantly enriched and adipocyte and macrophage colonies depleted compared to the unfractionated control (Herbertson and Aubin, 1997). In contrast, within the AP\textsuperscript{low} fraction of cells, adipocyte and macrophage colonies are consistently enriched (Herbertson and Aubin, 1997). We have used the RBM
stromal system, in combination with flow cytometric sorting, as a model for investigating the role that PDGF plays in osteoprogenitor differentiation.

4.2 Materials and Methods

Serum

Levels of PDGF in serum vary depending on how serum is prepared and the species used (e.g., PDGF levels in whole blood serum from normal humans (17.5 ± 3.1 ng/ml), baboons (2.7 ng/ml) (Bowen-Pope et al., 1984) and fetal bovine serum (0.929 ± 0.23 ng/ml) (Hyclone Defined Fetal Bovine Serum)). In these experiments, we used fetal bovine serum at a final concentration of 10%; even with the higher concentrations of PDGF reported (17.5 ng/ml for human whole blood serum), this would yield basal PDGF levels of approximately 1 - 2 ng/ml. In experiments in which Hyclone "defined fetal bovine serum" was used, the serum would contribute only .093 ng/ml PDGF, negligible compared to the additional concentrations (0.25 - 10 ng/ml) added. PDGF-BB is the major isoform seen in serum from most non-primate species (Bowen-Pope et al., 1989); accordingly, the porcine PDGF (R & D Systems, Inc.) used in these experiments contains only PDGF-BB homodimers.

Cell culture and plating

BM stromal cells from the femora of young 40 - 43-day-old, 110 - 120 gram, male Wistar rats were cultured essentially as previously described ((Maniatopoulos et al., 1988), see Chapter 2). After 7 d., adherent cells were recovered in a single cell suspension, and either counted electronically on a Coulter Counter (Coulter Electronics, Hialeah, FL) and plated at densities between 2 x 10^4 and 4 x 10^4 cells per 35 mm dish (Falcon) or stained and processed through the flow cytometric cell sorter (see Chapter 3) prior to plating. After plating, cells were cultured in growth medium plus porcine PDGF (1 - 10 ng/ml) in pulses as
indicated in the Figures or continuously as indicated for an average of 21 d., then stained (AP/von Kossa/toluidine blue, αNBE, and Sudan IV) as previously described in Chapter 2.

Quantitation

Quantitation of colony types was as previously described in Chapter 3. In addition, for analysis of multiple sets of data, the Wilcoxon signed rank test was used.

4.3 Results

As we reported previously (Herbertson and Aubin, 1995), dex markedly affects both the subpopulation make-up and osteogenic differentiation in RBM stromal cell cultures. While a small number of bone nodules form in cultures without dex, dex increases significantly the number of bone nodules and the number of macrophage colonies present, and decreases the proportion of cells expressing leukocyte common antigen. In all experiments reported here, PDGF was added to cultures also supplemented with dex.

Continuous exposure to PDGF

The continuous presence of PDGF (4 ng/ml) was mitogenic in RBM stromal cell cultures, significantly increasing late log phase growth rate and saturation density (see Figure 4.1). PDGF at the same concentration decreased the number of bone nodules, but concomitantly increased macrophage and adipocyte colony number (see Table 4.1), suggesting that the decrease in bone nodules is not due to generalized cell toxicity. PDGF dose response analysis was then undertaken for bone nodule (Figure 4.2), macrophage (Figure 4.3), and adipocyte (Figure 4.4) colony types. Multiple experiments were done over a three year period in regular and defined serum and data from all experiments are summarized in Table 4.2. PDGF had biphasic effects on bone nodule formation: the number of bone nodules was
stimulated by very low doses of PDGF (≤ 1 ng/ml in regular or defined serum experiments) and inhibited by higher doses (≥ 4 ng/ml); in multiple repeat experiments the intermediate concentrations either had no affect over control (Figure 4.2) or showed a sharper transition between stimulatory and inhibitory doses (e.g., Table 4.1). In three experiments in which complete dose response curves were available, the ID₅₀ for bone nodule formation was approximately 3 ng/ml PDGF (range in all experiments was approximately 2 - 10 ng/ml). In contrast, the continuous presence of PDGF consistently stimulated αNBE positive (previously confirmed to be macrophage colonies by specific antibody (ED2) labelling (Herbertson and Aubin, 1995)) colonies and adipocyte colonies at all concentrations tested in all experiments (see Tables 4.1, 4.2). Total macrophage colony number appeared to be maximally stimulated at PDGF concentrations of 2 - 2.5 ng/ml, however, the number of large macrophage colonies continued to increase dose dependently (see Table 4.1 and Figure 4.3) and may have overgrown and obscured smaller colonies. Likewise, adipocyte colonies appear to increase in a dose-dependent manner in all experiments (see Tables 4.1, 4.2 and Figure 4.3); in three experiments in which a plateau of adipocyte stimulation was seen (corresponding to the same experiments as for bone nodules above), the ED₅₀ was approximately 1.5 ng/ml.

**Pulse exposure to PDGF**

The development of bone nodules is characterized by a proliferative period followed by a period of morphologically and molecularly defined osteoblast differentiation and matrix deposition and mineralization (Liu et al., 1994; Lian and Stein, 1995; Aubin and Liu, 1996). To discriminate the developmental stages at which the biphasic effects of PDGF occurred, we pulse-treated cultures at different stages of the nodule formation process. 2 d. pulses with PDGF (4 ng/ml or 6 ng/ml) early during log phase growth (d. 1 - 3, d. 3 - 5) significantly stimulated bone nodule numbers. PDGF pulses during late log phase growth (d. 5 - 7 or d. 5 - 8) either increased (2/4 experiments) or did not alter (2/4 experiments)
bone colony formation. PDGF pulses later in the culture period (d. 7 - 9 onwards), when saturation density was reached and nodules were forming, tended to decrease or significantly decreased (d. 13 - 15) nodule formation (see Figure 4.5).

The effects of PDGF on RBM cells fractionated on the basis of AP expression

Flow cytometric sorting of 7 d. primary RBM stromal cell cultures was performed on the basis of AP expression with a mAb against AP (RBM 211.13). The resultant AP<sup>high</sup>, AP<sup>low</sup>, or control cells were plated to determine osteoprogenitor (giving rise to bone nodules), macrophage, and adipocyte distribution and frequency. As described in detail elsewhere (Herbertson and Aubin, 1997), within the AP<sup>high</sup> fraction, the number of AP-positive colonies and osteoprogenitors were significantly enriched while adipocyte and macrophage colonies were depleted compared to the unfractionated control. In contrast, within the AP<sup>low</sup> fraction of cells, adipocyte and macrophage colonies were consistently enriched (Figure 4.6). When these sorted populations were cultured with PDGF (4 ng/ml), results similar to those with unfractionated control populations were obtained. Specifically, in two separate experiments, the same treatment with PDGF (4 ng/ml. continuous exposure) significantly decreased bone nodules while significantly increasing macrophage and adipocyte colonies in the AP<sup>high</sup> fractions (see Figure 4.6). In the AP<sup>low</sup> fraction of cells, 4 ng/ml PDGF significantly decreased bone nodules in one experiment (see Figure 4.6) (no statistically significant difference was seen in the other), significantly increased adipocyte colonies, and significantly increased large macrophage colonies (often to an extent that colonies merged making counting of total colonies difficult) while slightly decreasing or not significantly affecting total macrophage colony numbers (see Figure 4.6).
Table 4.1  RBM stromal cells plated at $2 \times 10^4/35$ mm dish were grown for 21 d., with dex and the PDGF concentrations indicated present continuously from d. 1 - 21. PDGF had dose-dependent biphasic effects on bone nodule formation, while over the same concentration range both adipocyte and αNBE positive colonies increased. Results are means ± one standard deviation.

t tests versus PDGF 0 ng/ml: #p ≤ .05, *p ≤ .01, **p ≤ .001.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Bone nodules</th>
<th>Fat colonies</th>
<th>Total αNBE</th>
<th>Large αNBE</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF 0 ng/ml</td>
<td>25.7 (± 10.2)</td>
<td>1.0 (± 0.0)</td>
<td>8.8 (± 2.6)</td>
<td>0.0 (± 0.0)</td>
</tr>
<tr>
<td>PDGF 1 ng/ml</td>
<td>57.3 (± 16.4)#</td>
<td>9.0 (± 0.0)</td>
<td>57.5 (± 8.7)**</td>
<td>0.5 (± 1.0)</td>
</tr>
<tr>
<td>PDGF 2.5 ng/ml</td>
<td>42.5 (± 9.1)#</td>
<td>59.7 (± 13.1)**</td>
<td>136.3 (± 22.5)**</td>
<td>1.0 (± 0.8)#</td>
</tr>
<tr>
<td>PDGF 5 ng/ml</td>
<td>4.0 (± 7.4)#</td>
<td>63.7 (± 11.5)**</td>
<td>135.3 (± 4.2)**</td>
<td>3.3 (± 0.5)**</td>
</tr>
<tr>
<td>PDGF 10 ng/ml</td>
<td>0.3 (± 0.5)*</td>
<td>74.3 (±4.0)**</td>
<td>129.8 (± 9.7)**</td>
<td>6.0 (± 0.8)**</td>
</tr>
</tbody>
</table>
Table 4.2 Effect of continuous exposure to PDGF on RBM stromal populations

Net changes (stimulation = up arrow, inhibition = down arrow) with continuous exposure to PDGF at the doses indicated, compared to vehicle controls in RBM stromal cell cultures with dex. Changes indicated by the arrows were significant at minimally $p \leq .05$ as determined by $t$ tests, or the trend seen is indicated.

<table>
<thead>
<tr>
<th>PDGF ng/ml</th>
<th>Bone nodules</th>
<th>aNBE + colonies</th>
<th>Adipocyte colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>↑ 1/1</td>
<td>↑ 1/1</td>
<td>(not determined)</td>
</tr>
<tr>
<td>0.50</td>
<td>↑ 1/3 (trend ↑ 2/3)</td>
<td>↑ 2/3 (trend ↑ 1/3)</td>
<td>↑ 1/1</td>
</tr>
<tr>
<td>1</td>
<td>↑ 1/6 (trend ↑ 4/6) (trend ↓ 1/6)</td>
<td>↑ 5/6 (trend ↑ 1/6)</td>
<td>↑ 4/4</td>
</tr>
<tr>
<td>2 - 2.5</td>
<td>↓ 5/8 ↑ 1/8 (trend ↑ 2/8)</td>
<td>↑ 6/7 (trend ↑ 1/7)</td>
<td>↑ 4/4</td>
</tr>
<tr>
<td>4*</td>
<td>↓ 6/10 (trend ↓ 4/10)</td>
<td>↑ 8/10 (trend ↑ 2/10)</td>
<td>↑ 7/7</td>
</tr>
<tr>
<td>5-6</td>
<td>↓ 3/3</td>
<td>↑ 2/2</td>
<td>↑ 1/1</td>
</tr>
<tr>
<td>10</td>
<td>↓ 2/2</td>
<td>↑ 2/2</td>
<td>↑ 1/1</td>
</tr>
</tbody>
</table>

* Wilcoxon signed rank test of all experiments at 4 ng/ml PDGF show that overall patterns are significant, i.e., bone nodule numbers decrease ($p = .0020$), aNBE positive (macrophage) colony numbers increase ($p = .0020$), fat colony numbers increase ($p = .0156$).
Figure 4.1 Cell number (log scale) versus time cultured. The continuous presence of 4 ng/ml of PDGF (PDGF 4) significantly increased cell numbers present in RBM stromal cultures at days 4, 6, and 8 (late log phase growth) and the final saturation density when compared to the vehicle control (PDGF 0). t test: #p ≤ .05, *p ≤ .01.
Figure 4.2  The number of bone nodules present in RBM stromal cell cultures grown for 21d. in the continuous presence of dex and PDGF concentrations as indicated. PDGF 0.25 ng/ml significantly increased the number of bone nodules, while PDGF concentrations > 2 ng/ml dose-dependently decreased the number of bone nodules seen in dex treated cultures. t test versus vehicle control (PDGF 0): #p ≤ .05, *p ≤ .01, **p ≤ .001.
Figure 4.3 The number and size of αNBE positive (macrophage) colonies present in RBM stromal cell cultures grown for 21d. in the continuous presence of dexamethasone (dex) and PDGF concentrations as indicated. The total colony number increases start with PDGF 0.25 ng/ml and appear to plateau at PDGF concentrations ≥ 2 ng/ml, however, large colony numbers continue to increase. *t test versus vehicle control (PDGF 0) for the corresponding size or for total number: #p ≤ 0.05, *p ≤ 0.01, **p ≤ 0.001.
Figure 4.4 The number of adipocyte colonies present in RBM stromal cell cultures grown for 21d. in the continuous presence of dex and PDGF concentrations as indicated. PDGF concentrations > 1 ng/ml dose-dependently increased the number of adipocyte colonies. Since vehicle control (PDGF 0) had a mean and standard deviation of 0, one column tests versus a theoretical mean of 0 were used: #p ≤ .05, *p ≤ .01, **p ≤ .001.
Figure 4.5 The number of bone nodules present in RBM stromal cell cultures grown for 21d. in the continuous presence of dex and PDGF 4 ng/ml pulses as indicated. 2 d. PDGF pulses at d. 1 - 3, d. 3 - 5, and d. 5 - 7 significantly increased the number of bone nodules, while PDGF pulse at d. 13 - 15 showed a (small) decrease in the number of bone nodules. T test versus control (0 days exposed): #p ≤ .05, *p ≤ .01, **p ≤ .001.
Figure 4.6 Legend

The number of bone nodules present in RBM stromal cell cultures grown for 7d. primary culture, then sorted according to alkaline phosphatase (AP) expression (high expression = AP$^{\text{high}}$, low or negative expression = AP$^{\text{low}}$, unfractionated cells = control) and grown for 21d. in the continuous presence of dex and PDGF 4 ng/ml. Bone nodules are decreased significantly by PDGF in both AP$^{\text{high}}$ and AP$^{\text{low}}$ fractions; adipocyte colonies are significantly increased in all populations; total αNBE (macrophage) colonies increased in control and AP$^{\text{high}}$ populations. While the total αNBE colony number decreased in the AP$^{\text{low}}$ fraction in this experiment, the number of large colonies increased significantly, likely obscuring smaller colonies. t-test versus same condition without PDGF: #p ≤ .05, *p ≤ .01, **p ≤ .001.
4.4 Discussion

Our data suggest that PDGF affects both proliferation and differentiation in RBM stromal cell cultures. While the main purpose of our study was to investigate an effect of PDGF on osteoprogenitors and bone formation, we also monitored other lineages which are present in these cultures, regulated by some of the same factors and hormones (e.g., dex (Herbertson and Aubin, 1995)), and whose presence may modulate osteoblast lineage cell expression (Aubin, 1997, submitted). The mitogenic effects of PDGF on late log phase growth rate and saturation density of RBM stromal cultures, together with its ability to stimulate the numbers of macrophage and adipocytic lineage colonies, suggest that PDGF stimulates proliferation and differentiation in these lineages. In contrast, continuous exposure to PDGF decreased AP-positive colony and bone nodule number, suggesting that PDGF inhibits osteoprogenitor differentiation to mature bone-forming osteoblasts. The ability of pulse treatment with PDGF in early to late log phase growth to increase bone nodule numbers while pulse treatment late in the differentiation sequence inhibits bone nodule numbers suggests, however, that PDGF is stimulatory for progenitors at an early stage of osteoblast differentiation and is inhibitory at later stages. This is consistent with data on calvaria organ cultures in which the cells proliferating in response to PDGF appeared to be in the periosteal fibroblast and osteoprogenitor cell zones (Hock and Canalis, 1994), and with the suggestion that PDGF may stimulate a fibrous tissue, rather than bone tissue, phenotype (Marden et al., 1993). The biphasic nature of the effects of PDGF was also evident over different concentration ranges, with low doses stimulating and high doses inhibiting bone nodule formation. These in vitro studies may shed light on the conflicting data reported from PDGF treatments in vivo. Continuous exposure to PDGF may be considered analogous to studies with local administration of PDGF-BB (e.g., in rat craniotomy defects), which was found to inhibit bone regeneration (Marden et al., 1993). Since PDGF is rapidly cleared in rats (90% within 1 hr. (Cohen et al., 1990)), pulse exposures may mimic systemic
administration which has been shown to significantly increase osteoblast number (Mitlak et al., 1996)), as with early PDGF pulses in our culture system.

Previous studies reported that 24 - 48 hr. pulses with PDGF-BB (approximately 10 - 100 ng/ml) decreased AP activity in fetal rat calvaria cell cultures (Centrella et al., 1991; Pfeilschifter et al., 1992). Cassiede and colleagues found that a 48 hr. pulse of 5 ng/ml PDGF-BB on preconfluent rat marrow stromal cells had no significant effect on osteochondrogenic potential (as measured by bone nodule formation) and observed that a decrease in AP activity directly after pulse treatment is not predictive of subsequent osteochondrogenic potential on removal of the growth factor (Cassiede et al., 1996). We cannot account for why our data are different from Cassiede and colleagues, but since their pulse window (d. 4 - 6) falls between our d. 3 - 5 pulses (which were consistently stimulatory) and our late log phase growth (d. 5 - 7 or 5 - 8) pulses (which showed mixed increase/no difference result), it is possible that they treated at a particular developmental time/differentiation stage which is insensitive to a PDGF effect. Taken together with our biphasic PDGF concentration effects and the exquisitely sensitive timing required to target particular differentiation stages, the data argue for complex, multiphasic effects of PDGF on this bone cell lineage.

While dex concomitantly stimulated bone nodule formation and macrophage development in RBM stromal cell cultures (Herbertson and Aubin, 1995), continuous exposure to PDGF stimulated production of macrophage colonies but inhibited bone nodule formation. Both macrophages (Yan et al., 1993) and osteoblasts (Zhang et al., 1991) are known to express PDGF β-receptors, and thus both lineages should respond to porcine PDGF which is primarily PDGF-BB. Macrophages produce cytokines and growth regulating factors (eg., PGE₂, TNF, IL-6 (Horowitz and Gonzales, 1996); IGFBP-4 (Li et al., 1996)) which may affect osteoblastic cells, and inhibit stromal development (Goliaei et al., 1995). Since PDGF
strongly up-regulates IL-1 mRNA levels in murine macrophages (Yan et al., 1993), and the continuous presence of both IL-1β and IL-1α are known to inhibit bone formation (Ellies and Aubin, 1990), it seemed possible to us that the inhibitory effect of PDGF on bone nodules might be indirect and mediated through macrophages and IL-1 production. IL-1 has been shown to have biphasic effects on bone formation in RBM stromal cell cultures, and the dose response curve varies according to the endogenous IL-1 levels (Shadmand and Aubin, 1995), similar to the biphasic response we measured here with PDGF and the small shifts in dose responsiveness seen between independent RBM isolations. On the other hand, the fact that osteoblasts express PDGF-β receptors suggests that PDGF could have direct effects on osteoblast lineage cells. To test this hypothesis, we sorted RBM stromal cells on the basis of expression of AP. We showed previously that this resulted in populations 93-97% pure in the AP$^\text{high}$ fraction, a fraction significantly enriched for osteoprogenitors and depleted in adipocytes and macrophages, while the AP$^\text{low}$ fraction was significantly enriched in macrophages and fat cells (Figure 4.6, and Herbertson and Aubin, 1997). In two separate experiments, the effects of PDGF on osteoprogenitors (giving rise to bone nodules) in the AP$^\text{high}$ fractions appeared quantitatively similar to what we observed in the unfractionated control, suggesting a direct effect of PDGF on the osteoblast lineage. Of course, we cannot rule out that the fewer remaining monocyte-macrophage lineage cells in the AP$^\text{high}$ fraction are sufficient to effect a cytokine cascade mediating the PDGF response, but this seems unlikely given the similar fold-changes in bone in response to PDGF in populations with markedly different initial numbers and fold-changes in monocyte-macrophage cells. The reciprocal question of whether the effects of PDGF on macrophages are all direct is also of interest since osteoblastic cells are known to produce some of the factors thought to be involved in hematopoiesis including M-CSF (Shiina-Ishimi et al., 1986; Elford et al., 1987; Benayahu et al., 1992; Greenfield et al., 1993)). Again, there appears to be no direct relationship between the number of bone nodules and the number of macrophage colonies present in unfractionated or fractionated populations, suggesting that
the stimulatory effects of PDGF on macrophages are direct. In this regard, other authors have reported that PDGF-BB has a stimulatory effect on primitive pluripotential hematopoietic cells (preCFCmulti), mediated by up-regulation of IL-1 in marrow macrophages, and showed conclusively the presence of PDGF β-receptors on normal marrow macrophages (Yan et al., 1993).

Exposure to PDGF-BB has been reported to inhibit expression of differentiated phenotypes in certain other mesenchymal cell types, e.g., smooth muscle (Holycross et al., 1992). While chronic exposure to high concentrations inhibits osteogenesis, it is clear that low doses and pulsatile exposure of early progenitors leads to stimulation of differentiation to mature matrix-synthesizing osteoblasts. In addition, we consistently found a stimulation of adipocyte colony numbers between 1 - 10 ng/ml PDGF, which appears to be a novel observation. Indeed, PDGF has been reported to inhibit adipocyte differentiation in human female adipose tissue cultures, although the concentrations required to see inhibition were ≥ 10 ng/ml (Hauner et al., 1995), concentrations higher than we used here. It is not clear why Hauner et al. did not see a stimulatory effect although differences in the cultures and culture conditions used (including species differences - human versus rat; serum-free versus serum containing medium, and differences in anatomic sites (medullary versus non-medullary adipocytes)) are all possibilities. It is also interesting that PDGF is stimulatory to both adipocyte precursors and osteoblast precursors under at least some conditions, e.g., very low dose treatment, but that the responsiveness of the lineages diverges at higher concentrations such that PDGF stimulates adipogenesis while concomitantly inhibiting osteogenesis in the same cultures. Since we found that PDGF is mitogenic at early stages of culture during log phase growth, it is possible that progenitors for both lineages, including, e.g., a putative bipotential adipocyte-osteoblast progenitor (reviewed in (Gimble et al., 1996)), are comparably stimulated to undergo division, but that subsequent differentiation steps are modulated quite differently by this growth factor. In this regard, the differences in
sensitivity to PDGF (ED$_{50}$ for adipocyte colony stimulation, 1.5 ng/ml PDGF; ID$_{50}$ for bone nodule inhibition, 3 ng/ml PDGF, in the same experiments) of these lineages are notable and together with the marked differences in absolute number of adipocyte and osteoblast colonies, suggest that the stimulation in adipocyte formation is not at the expense of osteoblast formation, as suggested with some hormone treatment protocols, including 1,25(OH)$_2$D$_3$ treatment (reviewed in (Gimble et al., 1996; Aubin and Heersche, 1997)).

Our data suggest that the effects of PDGF on the differentiation of osteoprogenitors to mature osteoblasts in RBM stromal populations are biphasic, with both stimulation and inhibition of differentiation and bone nodule formation being observed, probably through the growth factor targeting different events early and late in the differentiation sequence. These complex effects, which occur concomitantly with modulation of other lineages present in the stromal environment, suggest that PDGF has pleiotropic effects on bone metabolism.
CHAPTER 5

Conclusions and Future Experiments
5.1 Conclusions and Future Experiments

The studies presented in this thesis were aimed at furthering knowledge on whether cell-cell interactions affect osteogenesis in the heterogeneous cellular environment of RBM cell cultures. To this end, RBM stromal cells were grown under conditions stimulating bone nodule formation, then histochemical and immunohistochemical staining, and flow cytometric analysis and sorting were used for description and analyses. Finally, the effect of PDGF was examined in this system using these techniques.

In Chapter 2, I began to elucidate and quantify some of the subpopulations present when RBM stromal cells are grown with or without dex under conditions favoring bone formation. Culture dishes were analyzed for cell counts, or stained with either histochemical or immunohistochemical stains, and colony types were quantitated, or cells were processed for flow cytometry. Dex significantly increased the number of AP positive colonies and von Kossa positive bone nodules, cNBE positive colonies, and ED2 positive (macrophage) colonies. The number of adipocyte foci was largely unaffected in these experiments. Flow cytometry confirmed colony counts and showed stimulation by dex of AP positive cells and macrophages, and in addition, the reduction of hemopoietic cells expressing leukocyte common antigen. These data show that when RBM stromal populations are grown under conditions stimulating osteoprogenitor differentiation and bone formation, the stromal subpopulation make-up, including expression of hemopoietic lineages, is markedly altered. Specifically, dex stimulated osteoprogenitor differentiation concomitant with promoting the growth and differentiation of macrophages. These data also showed for the first time the presence of macrophages in RBM stromal cultures grown under conditions favouring bone formation, and extended the information on the subpopulation make-up and heterogeneity under these culture conditions.
Since the presence of multiple cell types in RBM stromal populations may modulate or even mask, and complicate interpretation of, cytokine and hormone effects on the osteoprogenitors present, I looked for a method for purification of the osteoprogenitor population. In Chapter 3, I described experiments in which flow cytometric sorting of 7 d. primary RBM stromal cell cultures was performed on the basis of AP expression with an antibody against AP (RBM 211.13 (Turksen and Aubin, 1991)). The resultant \( \text{AP}^{\text{high}} \), \( \text{AP}^{\text{low}} \), or control cells were plated to determine osteoprogenitor, macrophage, and adipocyte distribution and frequency. Approximately 50% of osteoprogenitor cells (capable of giving rise to bone nodules) were lost during processing/sorting when compared to unsorted controls. Nevertheless, within the \( \text{AP}^{\text{high}} \) fraction, the number of AP positive colonies and bone nodules were significantly enriched compared to the unfractionated control; the increase in osteoprogenitor frequency ranged from approximately 2 to 100 fold. There were few assayable osteoprogenitors in either the \( \text{AP}^{\text{high}} \) or \( \text{AP}^{\text{low}} \) fractions in the absence of dex, suggesting that RBM stroma contains largely dex-dependent osteoprogenitor populations, and that dex may regulate osteoprogenitors subsequent to the upregulation of AP. Bone nodule numbers in either the \( \text{AP}^{\text{high}} \) or \( \text{AP}^{\text{low}} \) fraction did not follow a linear relationship with decreasing plating density. Adipocyte and macrophage colonies were depleted in the \( \text{AP}^{\text{high}} \) fraction of cells, but were enriched in the \( \text{AP}^{\text{low}} \) fraction. I thus showed that flow cytometric sorting of RBM stromal populations according to high or low AP expression is an effective technique for enrichment of AP positive colonies and osteoprogenitors capable of dividing and differentiating into bone nodule-forming cells, and should prove useful for further studies of the nature of the osteoprogenitors and their regulation in this tissue.

In Chapter 4, I combined the techniques of the previous two chapters to investigate the effects of PDGF in RBM stromal cell populations, again grown under conditions promoting growth and differentiation of osteoprogenitors to mature bone-forming osteoblasts. PDGF was mitogenic during late log phase growth and increased the saturation density of RBM
stromal cultures, as well as dose-dependently stimulating the numbers of macrophage and adipocytic lineage colonies, suggesting that PDGF stimulated proliferation and differentiation in these lineages. The continuous presence of PDGF over the same concentration range had biphasic effects on bone nodule formation, increasing bone nodules at low doses (≤ 1 ng/ml) and dose-dependently decreasing formation at ≥ 4 ng/ml. When present as a short-duration pulse in early to late log phase only, PDGF increased bone nodule numbers at all doses tested, but inhibited bone nodule formation late in the differentiation sequence, suggesting that PDGF is mitogenic during an early stage of osteoblast differentiation but inhibitory later. Comparison of control/mixed RBM stromal cultures with populations fractionated on the basis of AP expression suggested that the inhibitory effects of PDGF may be elicited by a direct action on osteoblast lineage cells. These complex effects on bone, which occur concomitantly with modulation of other lineages (macrophages and adipocytes) present in the stromal environment, suggest that PDGF has pleiotropic effects on bone metabolism.

The histochemical and immunohistochemical techniques described, along with the ability to fractionate RBM stromal populations on the basis of AP expression, have facilitated the characterization of the RBM stromal system, and will be of use when the effects of other cytokines or growth factors are analyzed in this system. We are currently limited in the rat system by a lack of other specific antibodies to early osteoprogenitors as recently described for human osteoblastic cells (Simmons and Torok-Storb, 1991; Haynesworth et al., 1992; Gronthos et al., 1994; Joyner et al., 1997), especially given that the specific macromolecules recognized by these antibodies are only beginning to be identified (see, e.g., (Bruder et al., 1997)) to allow preparation of rat-specific reagents. As other specific reagents are identified by antibody or molecular approaches, future sorting experiments can be designed to achieve even better enrichment for cells earlier in the osteoblast lineage. While the 93 - 97% purity obtained in our sorting experiments is comparable to other sorting and immunopanning
techniques, it has left open some ambiguity and necessitated some caution in interpretations of the data as discussed in Chapter 3 and Chapter 4. Nevertheless, the approaches have been established and they lead to reasonable cell yields, so that future experiments can all be aimed at improving the purity of the osteoblast lineage rather than development of techniques. Methods for improving purity could include double sorting with one specific antibody, use of both positive and negative immunoselection in sorting, and/or a combination of sorting with immunopanning techniques. Likewise, as antibodies become available recognizing other rat early hemopoietic cells, it will become possible to identify more precisely and manipulate the leukocyte antigen positive cells, whose numbers are also markedly altered by dex in osteogenic RBM cultures, to determine more clearly their role/interactions with osteoprogenitors in this system. The ability to identify and then fractionate the subpopulations present in RBM stromal cell populations represents an important step towards understanding the cell-cell interactions which affect bone formation in BM.
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